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(71) Applicant: DANA-FABER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US).

(72) Inventors: REINHERZ, Ellis ; HUSSEY, Rebecca ; 113 South Great Road, Lincoln, MA 01773 (US). SODROSKI, Joseph ; 10 Ashland Place, Medford, MA 02155 (US). RICHARDSON, Neil (deceased) ; CLAYTON, Linda, K. ; 48 Cedarwood Road, Jamacia Plain, MA 02130 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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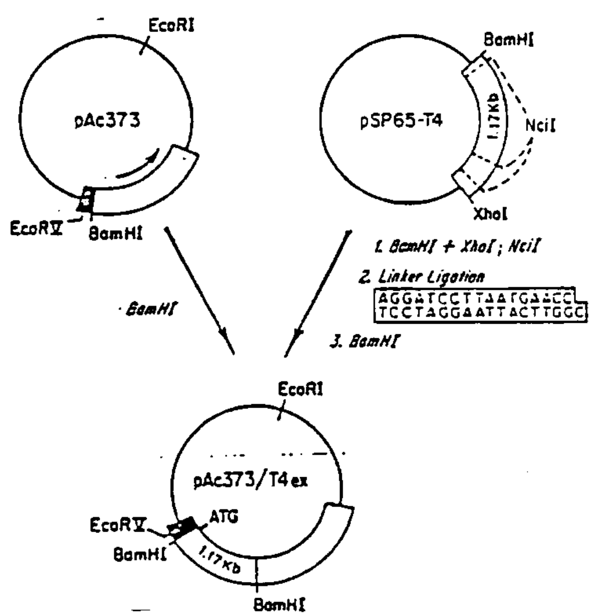
(54) Title: SOLUBLE HUMAN CD4 FRAGMENTS AND USES THEREFOR



(57) Abstract

Modified soluble human CD4 fragments which are capable of binding HIV gp120 envelope protein and which do not interfere with the function and proliferation of T lymphocytes not infected by HIV, DNA encoding such fragments and uses therefor. Such fragments can be used for diagnostic, therapeutic and preventive purposes.

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SOLUBLE HUMAN CD4 FRAGMENTS AND USES THEREFOR

Description

Background

The CD4 (T4) molecule, which is a surface glycoprotein on a subset of T lymphocytes (referred to as T4 lymphocytes) is involved in Class II (Ia) MHC recognition and appears to be the physiological receptor for one or more monomorphic regions of class II MHC. Meur, S. et al., Proceedings of the National Academy of Sciences, U.S.A., 79:4395-4399 (1982); Biddison, W. et al., J. Exp. Med., 156:1065-1076 (1982); Gay, D. et al., Nature, 328: 626-629 (1987).

Human CD4 is also the receptor for the gp120 envelope glycoprotein of the human immunodeficiency virus (HIV) and is essential for virus entry into the host cell, and for membrane fusion, which both contribute to cell-to-cell transmission of the virus and to its cytopathic effects. Klatzmann, D., et al., Science, 225: 59-63 (1984); Dalgleish, A.G., et al., Nature, 312: 763-766 (1984); Sattentau, Q., et al., Science, 234: 1120-1123 (1986); McDougal, J.S., et al., J. Immunol., 137: 2937-2944 (1986); McDougal, J.S., et al., Science, 231: 382-385 (1986); Maddon, P.J., et al., Cell, 47: 333-348 (1986); Sodroski, J., et al., Nature, 322: 470-474 (1986); Lifson, J., et al., Nature, 323: 725-728 (1986). Sequence analysis of CD4 has suggested an evolutionary origin from a structure with four immunoglobulin-related domains. Clark, S., et al., Proc. Natl. Acad. Sci., 84: 1649-1653 (1987); Littman, D.R., et al., Nature, 325:

453-455 (1987). Only the two NH_2-terminal domains are required to mediate HIV gp120 binding. Traunecker, A., et al., Nature, 331: 84-86 (1988); Berger, E.A., et al., Proc. Natl. Acad. Sci. USA, 85: 2357-2361 (1988); Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA, in press.

Considerable effort has been expended in studying the CD4-gp120 interaction and in trying to interfere with or inhibit that interaction, in an attempt to provide a means by which the life threatening effects of HIV infection can be slowed or reversed. Several groups have focused their efforts on the ability of soluble CD4 (T4) protein to interfere with infection of cells by HIV and its subsequent effects. Hussey, R.E. et al., Nature, 331:78-81 (1988); Fisher, R.A. et al., Nature, 331:76-78 (1988); Deen, K.C. et al., Nature, 331:82-84 (1988); Traunecker, A. et al., Nature, 331:84-86 (1988). A means by which to prevent HIV infection of T4 lymphocytes (i.e., helper and inducer T lymphocytes), which make up approximately 60-80% of the total circulating T lymphocyte population, would be of great value, particularly in light of the fact that HIV infection of such cells can cause total collapse of the immune system. Curran, J. et al., Science, 229:1352-1357 (1985); Weiss, R. et al., Nature, 324:572-575 (1986).

Disclosure of the Invention

The present invention relates to soluble human CD4 (T4) fragments which bind to the HIV gp120 envelope protein (HIV gp120); to soluble human CD4 fragments whose ability to bind to the HIV gp120

envelope protein has been altered; to DNA encoding such types of human CD4 fragments; to methods of using soluble human CD4 fragments in interfering with infection of cells by HIV; to methods of modifying the amino acid sequence of soluble human CD4 fragments; and to methods of modifying or altering the ability of soluble human CD4 fragment to bind HIV gp120. (CD4 and T4 are used herein interchangeably).

Soluble human CD4 fragments include none of the hydrophobic transmembrane region of CD4 or only a portion (generally six amino acids or less) of the hydrophobic region which does not prevent solubilization of the fragments. As a general class or category, soluble human CD4 fragments which are capable of binding with HIV gp120 are referred to as biologically active soluble human CD4 fragments. As explained below, biologically active soluble human CD4 fragments can be modified, with the result that the amino acid sequence differs in some way from that of the corresponding portion of naturally-occurring CD4. All such fragments (i.e., those which correspond in amino acid sequence with the naturally-occurring CD4 and those which are modified) which are capable of binding HIV gp120 are included within the term biologically active soluble human CD4 fragments, as used herein. However, for ease of discussion, soluble CD4 fragments which have in some way been modified as to amino acid sequence are referred to as biologically active, modified soluble CD4 fragments. Those fragments whose HIV gp120 binding ability has been changed are referred to as modified soluble CD4 fragments with altered HIV gp120 binding ability. In those cases in which HIV gp120

binding ability is increased or enhanced, such fragments can be referred to as biologically active, modified soluble CD4 fragments with enhanced HIV gp120 binding ability. Conversely, those whose binding ability is reduced can be referred to as modified soluble CD4 fragments with reduced HIV gp120 binding ability.

Biologically active soluble human CD4 fragments can be modified in several different ways. The amino acid sequence of soluble human CD4 can be: 1) truncated; 2) altered by means of substitution(s) in, deletion(s) from and/or addition(s) to the amino acid sequence; or 3) both truncated and altered. These three types or classes of fragments can be referred to, respectively, as truncated, altered, and truncated/altered.

Biologically active soluble CD4 fragments of the present invention have the ability to bind to HIV. They will, therefore, also have the capacity to prevent infection of human T-lymphocytes by HIV and to prevent formation of the human T-lymphocyte syncytia which are thought to play a role in transmission of HIV from cell to cell.

Such biologically active soluble CD4 fragments can be used for diagnostic, therapeutic and preventive purposes. For example, they can be used to determine the presence or absence of HIV gp120 in a biological sample (e.g., blood, urine, saliva, semen) and, thus, to determine whether HIV is present in the sample or not. In addition, they can be used to treat individuals infected with HIV, in vivo (e.g., by administration to infected individuals). They can also be used prophylactically. That is,

they can be administered to individuals at risk for HIV infection. Further, they can be used to prevent infection by HIV by, for example, being coated onto materials used as barriers against introduction of the virus (e.g., condoms, spermicides, garments, containers for collecting, processing or storing blood, etc.).

Modified soluble CD4 fragments with altered HIV gp120 binding ability can be used for diagnostic, therapeutic and preventive purposes. They can be used in a similar manner as described above for use of biologically active soluble human CD4 fragments.

Brief Description of the Drawings

Figure 1 is the nucleotide sequence of T4 SEC1 cDNA (referred to as the $T4_{ex1}$ sequence), which encodes 370 amino acids of soluble CD4 protein (referred to as $T4_{ex1}$). Modifications in cDNA and in the encoded CD4 protein are indicated by the boxed areas; each box represents the nucleotide triplet and encoded amino acid at which the modification is made.

Figure 2 is a schematic representation of the construction of an expression vector of the present invention.

Figure 3 is a schematic representation of the method by which the biologically active, modified soluble CD4 fragments of the present invention are produced.

Figure 4 is a graphic representation of the effects of the soluble CD4 fragments of the invention

and the effects of control proteins on viral protein replication.

Figure 5 is a graph illustrating the lack of inhibition of CTL effector function by the soluble CD4 fragments of the invention.

Figure 6 is a bar graph illustrating the lack of inhibition of proliferation of normal helper T-lymphocytes by the soluble CD4 fragments of the invention.

Figure 7 is a schematic representation of the structure of native and recombinant CD4 proteins. Figure 7A is a representation of the native CD4 protein structure derived from the cDNA sequence of Figure 1. Numbers in parentheses indicate the four putative extracellular domains; the S at 16, 84, 130, 159, 303 and 345 indicates the position of cysteine residues; Tm: transmembrane; Cty: cytoplasmic region. Figure 7B is a schematic representation of the $T4_{ex1}$ protein. Figure 7C is the complete amino acid sequence of the $T4_{ex1}$ protein.

Figure 8 is a schematic representation of CD4 protein $T4_{ex1}$ showing the four immunoglobulin-like domains, three disulfide bonds and two potential glycosylation sites. Numbering of amino acids is according to Hussey et al., Nature, 331:78-81 (1988). The positions of 16 mutations (see the Table) are represented below the line. The triangle indicates a stop codon introduced by site directed mutagenesis to create a protein containing only the first 182 amino acids.

Figure 9 shows results of anti-CD4 immunoprecipitation and anti-gp120 co-precipitation of $T4_{ex1}$ and a truncated 182 amino acid version of

CD4 from supernatants of ^{35}S-cysteine labelled Cos-1 cells transfected with the CD4 constructs. Lane 1, immunoprecipitation of supernatant from Cos-1 cells transfected with the $T4_{ex1}$ containing plasmid and immunoprecipitated with anti-T8 (21Thy2D3) (control); lane 2, immunoprecipitation of supernatant from Cos-1 cells transfected with the 182 amino acid truncation using the control anti-T8 antibody; lane 3, immunoprecipitation of $T4_{ex1}$ with anti-CD4 antibody (19Thy5D7); lane 4, immunoprecipitation of the 182 amino acid truncation of $T4_{ex1}$ with anti-CD4; lane 5, co-precipitation of $T4_{ex1}$ with anti-gp120 (DuPont) in the presence of gp120; lane 6, co-precipitation of $T4_{ex1}$ with anti-gp120 in the absence of gp120; lane 7, co-precipitation of the 182 amino acid truncation of $T4_{ex1}$ with anti-gp120 in the presence of gp120. All samples are run non-reduced. The molecular weight markers are phosporylase B (97.4KD), bovine serum albumin (69KD), ovalbumin (46KD), carbonic anhydrase (30KD), lactoglobulin A (18.4KD).

Figure 10 shows results of anti-CD4 immunoprecipitation of ^{35}S-cynsteine labelled supernatants from Cos-1 cells transfected with $T4_{ex1}$, M5, M10, M7 and M3. Precipitations were carried out in the presence (+) or absence (-) of gp120.

Detailed Description of the Invention

The present invention relates to soluble human CD4 fragments which bind to HIV gp120, as well as to soluble human CD4 fragments having altered gp120 binding ability; to DNA encoding soluble human CD4 fragments; to methods of making soluble human CD4 fragments and to methods of using soluble human CD4

fragments of the present invention to interfere with HIV infection of cells. In particular, it relates to soluble human CD4 fragments in which the amino acid sequence is the same as that of the corresponding region of naturally-occurring human CD4; to soluble human CD4 fragments in which the amino acid sequence has been modified, with the result that their amino acid sequences differ, as described below, from that of the corresponding region of naturally-occurring human CD4; to soluble human CD4 fragments whose binding ability is different from that of naturally-occurring human CD4 or the corresponding human CD4 fragment and to DNA encoding such soluble human CD4 fragments.

Soluble human CD4 fragments of the present invention include none of the hydrophobic transmembrane region of naturally-occurring CD4 or contain a portion of the hydrophobic region which is sufficiently short (i.e., generally six amino acids or less) that it does not prevent solubilization of the fragments. Soluble human CD4 fragments capable of binding HIV gp120 are referred to herein as biologically active soluble human CD4 fragments. Biologically active soluble human CD4 fragments are long enough (e.g., 10 amino acids or longer) that they are able to bind effectively to HIV gp120. Fragments need not exhibit total homology with the amino acid sequence of the corresponding region of human CD4. Rather, they must have sufficient homology to bind to HIV gp120.

In addition, biologically active soluble human CD4 fragments of the present invention are able to exert an anti-HIV effect, as a result of binding HIV

gp120, without interfering with the function or proliferation of human T-lymphocytes not infected with HIV. That is, biologically active soluble human CD4 fragments of the present invention have been shown, as described below, to prevent infection of human T-lymphocytes by HIV and to inhibit HIV envelope-induced syncytium formation and HIV replication without inhibiting Class II MHC recognition events (i.e., without inhibiting CTL effector function), even at high concentrations, and without having a discernible effect on Class II-directed physiologic T cell responses.

As used herein, the term soluble human CD4 fragments includes all soluble human CD4 fragments (i.e., those in which the amino acid sequence corresponds to that of naturally-occurring human CD4 and those in which modification of amino acid sequence has been made) capable of binding HIV gp120. Biologically active soluble human CD4 fragments in which the amino acid sequence has been modified are referred to biologically active, modified soluble human CD4 fragments. Fragments whose HIV gp120 binding ability has been changed (with the result that it is different from that of the corresponding or equivalent portion of naturally-occurring CD4) are referred to as modified soluble human CD4 fragments with altered HIV gp120 binding ability.

Biologically active, modified soluble human CD4 fragments of the present invention differ from that of soluble human CD4 (e.g., from the sequence represented in Figures 1 or 7C) in that the amino acid sequence: 1) is truncated; 2) has been altered as a result of deletion(s) from, substitution(s) in

and/or addition(s) to the amino acid sequence of human CD4; or 3) it is truncated and the truncated form or portion includes deletion(s) from, substitution(s) in and/or addition(s) to the amino acid sequence which occurs in the corresponding portion or segment.

Modified soluble human CD4 fragments having altered HIV gp120 binding ability are modified soluble human CD4 fragments in which the amino acid sequence of soluble human CD4 is altered at a selected site or sites in such a manner that the resulting CD4 fragment has HIV gp120 binding ability or affinity less than that of the corresponding (unaltered) soluble human CD4 fragment, or HIV gp120 binding ability or affinity greater than that of the corresponding (unaltered) human CD4 fragment. Such fragments are referred to, respectively, as modified soluble human CD4 fragments with diminished HIV gp120 binding ability and modified soluble human CD4 fragments with enhanced HIV gp120 binding ability.

In particular, CD4 fragments with altered HIV gp120 binding ability differ from soluble human CD4 fragments in that the amino acid sequences of the CD4 fragments with altered gp120 binding ability are different from the amino acid sequence of the soluble CD4 protein at a site or sites which have been found to be critical for gp120 binding. Until the present time, it has not been possible to selectively alter gp120 binding ability of soluble CD4 fragments because sites critical to gp120 binding had not been identified. Such critical sites have now been identified by means of oligonucleotide-directed mutagenesis and have been found to occur in domain I

and domain II of human CD4 protein, suggesting that the HIV gp120 binding site is complex and involves both of the NH_2-terminal domains. Modifications of the T4 cDNA, as it is represented in Figure 1, have been made and the encoded CD4 fragments expressed. Resulting CD4 fragments have been shown to have altered gp120 binding ability in vitro; in these instances, gp120 binding ability has been abrogated. Modifications at these same sites, and at other, as yet unidentified, sites, as described herein, can similarly be made to enhance gp120 binding ability, as well as to reduce or turn down (but not eliminate) gp120 binding ability.

The following is a brief description of the methods by which soluble human CD4 fragments of the present invention were produced; these are subsequently described in detail in the Examples.

Production of Biologically Active Soluble Human CD4 Fragments

Construction of plasmids used to produce soluble human CD4 fragments can best be described with reference to Figure 2. As shown in Figure 2, plasmid vector $PAc373/T4_{ex}$, which contains the truncated CD4 gene, was constructed from plasmids pAc373 and pSP65-T4. As described in detail in Example 1, a secreted form of the CD4 molecule was produced by releasing the CD4 cDNA insert contained in pSP65-T4. The CD4 cDNA insert was digested with NciI to produce a 1.17Kb fragment which lacks the ATG start codon and terminates just before the transmembrane region. The 1.17Kb fragment was ligated to a synthetic linker, with the result that either 371 residues ($T4_{ex1}$) or

370 residues ($T4_{ex2}$) of the mature extracellular segment would be preserved.

Recombinant plasmids were produced and two (designated pAc373/$T4_{ex1}$ and pAc373/$T4_{ex2}$) were characterized in detail. The truncated CD4 cDNA constructs were integrated into the Autographa californica nuclear polyhedrosis virus (AcNPV) genome by homologous recombination, using known methods. Smith et al., Proc. Natl. Acad. Sci., U.S.A., 82:8404-8408 (1985). Baculovirus stocks were used to infect Spodoptera frugiperda (SF9) cells, which are publicly available. Subsequently, SF9 cells infected with the recombinant baculovirus containing the $T4_{ex}$ cDNAs or wild type AcNPV were cultured in ^{35}S-methionine and products were examined by SDS-PAGE, followed by autoradiography.

The $T4_{ex1}$ polypeptide was shown to be the major secreted product of SF9 cells infected with the $T4_{ex1}$ recombinant baculovirus. The predominant ^{35}S labelled protein band in SDS-PAGE analysis of supernatants from SF9 cells obtained 54 hours after $T4_{ex1}$ recombinant baculoviral infection was a 50KD band under reducing conditions. No CD4 material was precipitated from supernatants of wild type AcNPV-infected cells or detectable in the total supernatant.

Each of two representative $T4_{ex2}$ preparations yielded a protein that migrated under reducing conditions with a molecular weight of 51KD. The different mobility (from that observed for $T4_{ex1}$ protein) was not unexpected, given that $T4_{ex2}$ contains 17 additional carboxy terminal amino acids derived from fusion with the polyhedrin gene.

As described in Example 1, further analysis demonstrated that the 50KD $T4_{ex1}$ and 51 KD $T4_{ex2}$ proteins were the products of the CD4 gene. The soluble CD4 proteins produced in the baculovirus system were shown to bind to the HIV gp120 exterior glycoprotein, as described in Example 1, using two reciprocal co-precipitation experiments. Inhibition of HIV gp120 binding to CD4 + B4 lymphocytes by the $T4_{ex1}$ protein was also demonstrated, as were inhibition of HIV replication and inhibition of HIV envelope-induced syncytia, by the $T4_{ex1}$ and the $T4_{ex2}$ proteins (Example 1). As mentioned previously and as described in detail in Example 1, these effects were shown to be produced by the CD4 fragments without having a discernible effect on Class II MHC recognition events (e.g., they failed to inhibit CTL effector function), even in high concentrations. In addition, the soluble CD4 fragments were shown to have no discernible effect on Class II-directed physiologic T cell responses; they were shown to have no effect on proliferation of the T4+ tetanus toxoid specific Class II MHC restricted helper T cell clone CTT7 (Example 1).

Another approach was used to further analyze the specific physical interaction between $T4_{ex}$ proteins or their derived peptide fragments and HIV gp120. This approach is described in detail in Example 2. Briefly, this method made use of size fractionation of SDS-PAGE, followed by electroblotting of the $T4_{ex2}$ protein onto polyvinylidine difluoride membranes. Results of this work (see Example 2) showed that the single band of $T4_{ex2}$ protein at 50KD MW, when electrophoresed unreduced, was capable of binding HIV

gp120 strongly. Conversely, identical quantities of $T4_{ex2}$, reduced or reduced and amidomethylated, did not bind HIV gp120. Identical results were obtained with $T4_{ex1}$ proteins.

Enzymic fragmentation of the purified $T4_{ex}$ protein was also carried out. Results of papain digestion showed the presence of a fragment with a mobility of 28KD which binds HIV gp120. It was shown to bind HIV gp120 with the same efficiency as the parent $T4_{ex1}$ protein and to be an intact polypeptide chain derived from the amino terminal region of the $T4_{ex1}$ protein. Similar experiments using trypsin fragmentation of $T4_{ex2}$ were also carried out to further define the nature of the HIV gp120 binding fragments, as described in Example 2.

Modification of Biologically Active Soluble Human CD4 Fragments

The cDNA sequence which encodes 370 amino acids of mature CD4 protein (T4 SEC1 cDNA) is represented in Figure 1, as is the deduced amino acid sequence of the encoded CD4 protein. Modifications of the T4 cDNA as represented in Figure 1 have been made and the encoded soluble CD4 fragment expressed. Resulting CD4 fragments have been shown to bind to HIV gp120 in vitro, as demonstrated by the ability to detect a complex between HIV gp120 and soluble CD4 proteins in solution.

As explained previously and in Example 1, biologically active soluble human CD4 fragments encoded by the nucleotide sequence of Figure 1 bind HIV gp120 and interfere with HIV infection of T cells without interfering with the function or

proliferation of human T lymphocytes which are not infected with HIV.

Briefly, biologically active, modified soluble CD4 fragments are produced as described in the following paragraphs and as presented schematically in Figure 3. Detailed description of production of such fragments is presented in Example 3.

DNA encoding a soluble CD4 fragment is produced, either by using recombinant DNA techniques, such as excising it from a vector containing cDNA encoding such a fragment (see Example 1) or by synthesizing DNA encoding a soluble CD4 fragment mechanically and/or chemically, using known techniques.

In either case, the DNA obtained encodes a soluble CD4 fragment, capable of binding to the gp120 envelope protein of HIV <u>in vitro</u>, which includes none of the hydrophobic transmembrane region of CD4 or a portion of that region (generally six amino acids or less) small enough that it does not prevent solubilization of the fragment. In addition, the CD4 fragment is long enough (e.g., 10 amino acids or more) to bind effectively to HIV gp120 envelope protein.

Templates for subsequent mutagenesis are produced, using the CD4 fragment-encoding cDNA or DNA. As described below, this can be carried out using a single-stranded bacteriophage cloning vehicle, such as M13. This results in production of single-stranded DNA homologous to only one of the two strands of the DNA encoding the soluble CD4 fragment. The resulting single-stranded DNA is used as a template for producing the biologically active, modified soluble CD4 fragments, as follows:

Oligonucleotides are produced, such that their sequence includes a base change (or changes) which, when incorporated into the nucleotide sequence of DNA subsequently used for the production of soluble CD4 fragments, results in a change in the encoded CD4 protein (i.e., different from that encoded by the nucleotide sequence of Figure 1). Such oligonucleotides are produced using standard methods. Oligonucleotides having a base change or base changes are referred to as mutagenized or mutant oligonucleotides.

The mutant oligonucleotide produced in this manner is hybridized to (e.g., by being kinased) the template produced as described above, to produce a template-mutant oligonucleotide complex, referred to as a mutant primer/template. The mutant primer/template is used for the production of a second strand of DNA, using well- known techniques. For example, synthesis of the second DNA strand is carried out by the Klenow fragment of DNA polymerase in the presence of dCTPαS. Taylor, J.W. et al., Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. et al., Nucleic Acids Research, 13:8764-8785 (1985); Nakayame, K. and F. Eckstein, Nucleic Acids Research, 14:9679-9698 (1986). The resulting strand of DNA contains a modification (or modifications) in the nucleotide sequence of T4 cDNA (i.e., is different from the nucleotide sequence represented in Figure 1) and is referred to as a mutant strand.

Unreplicated single-stranded DNA is removed and the double-stranded DNA is nicked with a selected restriction enzyme (e.g., NciI, which does not cut phosphorothioate DNA and, thus, does not cut the new

DNA strand containing dCTPαS or the mutant strand). Nicked, nonmodified DNA is removed by digestion with another enzyme, such as exonuclease III. The resulting gapped DNA is repolymerized and, because the mutant strand serves as the template for repolymerization, the mutation or modification is copied into both strands.

Once produced, the double-stranded DNA, in which both strands contain the mutation or modification encoding the corresponding modification in the amino acid sequence of the soluble CD4 fragment is introduced into a competent host cell, such as a competent bacterial host (e.g., by transformation). The resulting plaques are grown and DNA contained in them is isolated, using known techniques, and sequenced to confirm the presence of the mutation.

The mutated DNA produced in this manner is excised from the M13 vector containing it, introduced into a suitable expression vector, such as CDM8, and transfected into an appropriate host cell, such as Cos cells, in which it is expressed. Aruffo, A. and B. Seed, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). As a result, mutant CD4 proteins can be assayed, using known techniques. The vector-insert ligation mixture is introduced into competent host bacteria, such as the publicly available *E. coli* MC1061P3, and radiolabelled T4 DNA is used to identify CDM8 containing mutant T4 cDNAs.

Production, in Cos cells transfected with the vector containing mutant T4 cDNA, of modified soluble CD4 fragments capable of binding HIV (i.e., biologically active, modified soluble CD4 fragments)

is subsequently assayed, using known techniques described below.

As a result of this procedure, double stranded DNA encoding a biologically active, modified soluble CD4 fragment is produced, the encoded CD4 fragment is expressed and its ability to bind the HIV gp120 envelope protein is assessed.

An alternative approach to producing a biologically active, modified soluble human CD4 fragment of the present invention is to use peptide synthesis to make a peptide or polypeptide having the amino acid sequence of such a fragment.

This aspect of the subject invention will now be illustrated with reference to a specific modification, which is described in detail in Example 3 and production of which is represented in Figure 3. It is to be understood, however, that this is not meant to be limiting in any way and that other modifications can be made, using known techniques and the method of the present invention.

<u>T4 cDNA and Templates for Mutagenesis</u>

As represented in Figure 1, the $T4_{ex}$ cDNA was excised from the plasmid vector pAc373/$T4_{ex}$, using the restriction enzyme BamHI. The ends of the fragments were blunted with DNA polymerase I and the fragment was ligated to XbaI linkers. The ligated fragment was cut with XbaI, excess linkers were removed and the linkered fragments were ligated to Xba-cut M13 (replicative form). M13 is a single-stranded bacteriophage cloning vehicle which has a closed circular DNA genome approximately 6.5Kb in size. Messing, J. and J. Viera, <u>Gene</u>, <u>19</u>:269-276

(1982). It is useful as a cloning vehicle in this context because infected cells release phage particles which contain single-stranded DNA which is homologous to only one of the two strands of cloned DNA and which can be used as a template. The resulting ligation mixture was transformed into competent TG1 host bacteria, which were plated out. The plaques were screened, using T4 oligonucleotides. Plaques hybridizing to sense oligonucleotides were selected and grown up to produce single-stranded M13 templates for mutagenesis.

Mutagenesis

Mutagenesis was carried out by the protocol which is marketed by Amersham and is based on the method of Eckstein (See Example 3).

Oligonucleotides whose sequence included a base change which, when incorporated, produced an amino acid change in the encoded CD4 protein (different from that encoded by the cDNA protein of Figure 1) were produced, using standard methods. In this case, a truncation of the CD4 molecule was introduced at amino acid #183. The normal T4 cDNA sequence is G-AAG-GCC-TCC-AGC-ATA-G (see Figure 1). An oligonucleotide having the sequence 5'G-AAG-GCC-TAA-AGC-ATA-G was synthesized. The difference in the two sequences is underlined. The serine encoded by the TCC of the normal T4 cDNA was modified to a stop codon (TAA) and the encoded modified protein terminated at this point (resulting in a cDNA fragment in which the terminal triplet is GCC and the terminal amino acid is alanine).

Production of Double-Stranded DNA

The modified oligonucleotide was kinased and hybridized to M13 T4 template, which served as a template for synthesis of a second strand of DNA, by the Klenow fragment of DNA polymerase in the presence of dCTPαS. Taylor, J.W. et al., Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. et al., Nucleic Acids Research, 13:8764-8785 (1985); Nakayame, K. and F. Eckstein, Nucleic Acids Research, 14:9769-9698 (1986). This resulted in production of a strand of DNA (the second strand) containing a modification of the normal T4 cDNA nucleotide sequence (i.e., the sequence as represented in Figure 1). This modified strand is referred to as a mutant strand. Unreplicated single-stranded DNA was removed and the double-stranded DNA was nicked with the restriction enzyme, NciI. Because NciI does not cut phosphorothioate DNA, the new strand containing dCTPαS and the mutation was not nicked. The nicked, nonmodified DNA was removed by digestion with another enzyme (exonuclease III).

The gapped DNA was repolymerized using DNA polymerase I in the presence of T4 DNA ligase. Because the mutant strand served as the template, the mutation or modification was copied into both strands. The resulting double-stranded DNA was introduced into competent TG1 by transformation. Mandel, M. and A. Higa, Journal of Molecular Biology, 53:154 (1970). Derived plaques were grown up and single stranded and replicative form DNAs were isolated. The DNA was sequenced to confirm the presence of the mutation.

Mutated DNA (DNA including the mutation introduced as a result of the DNA synthesis using the modified oligonucleotide, as described above) was excised from the replicative form of DNA with Xba and ligated to vector CDM8 which had been cut by Xba. The CDM8 vector is expressed in Cos cells upon transfection. Cos cells are a monkey kidney cell line, which have been transformed by simian virus 40 (SV40) DNA which includes the functional early gene region, and thus constitutively expresses the SV40 large T antigen, but has a defective origin of viral DNA replication. Gluzman, Y. et al., Cell, 23: 175-182 (1981). The CDM8 vector containing mututed DNA was transfected into Cos cells, in which it was expressed, thus making it possible to assay mutant CD4 proteins. The vector-insert ligation mixture was introduced into competent MC1061P3 host bacteria and CDM8 containing mutant T4 cDNAs, were identified by hybridization to radiolabelled T4 DNA. Ausubel, F.M. et al. (ed.), Current Protocols in Molecular Biology, Greene Publishing Associates, p. 1.4.9 (1988), Seed, B. and A. Aruffo, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). Restriction enzyme analysis of mini-prep DNAs was used to determine the proper orientation of the insert in the CDM8 vector.

Determination of Ability of Modified Soluble CD4 Fragments to Bind HIV

Cos cells transfected with the CDM8 vector containing mutant T4 cDNA were assayed for production of modified soluble CD4 proteins capable of binding

HIV, as described briefly in the following sections and in detail in the Exemplification.

Cos cells transfected with the mutant T4 cDNA-containing CDM8 vector were processed in order to produce dialyzed supernate, which was precleared with control rabbit anti-T cell receptor IgG coupled to a Sepharose support in order to minimize non-specific binding.

The precleared supernate was immunoprecipitated with a monoclonal anti-CD4 antibody (19Thy5D7) coupled to a Sepharose support. 19Thy5D7 is an antibody against a T4 epitopic site which competes with HIV for binding of gp120. Thus, binding of a component of the supernate to 19Thy5D7 is suggestive of the presence in the supernate of a component capable of binding HIV.

The ability of the modified soluble CD4 fragments produced in this manner to bind to the HIV exterior gp120 glycoprotein can be directly determined as follows:

Labelled and dialyzed Cos supernates determined to contain optimal levels of recombinant, secreted CD4 protein will be taken for co-precipitation studies. For example, 67 ng of gp120 (1 ul at 67 ug/ml in PBS/0.1% BSA) can be added to 0.5 ml of Cos supernates. As a control, no addition is made to a second 0.5 ml aliquot of supernate. After a 2 hour incubation at 37°C, 500 ng of monoclonal anti-gp120 are added to both supernates, followed by rabbit anti-mouse IgG coupled to Sepharose 4B (10 ul). The samples are then rotated for 2 hours at 4°C. The beads are then wash twice with 100 ul cold PBS and eluted with non-reducing SDS sample buffer (30 ul).

Aliquots are run on 12.5% non-reduced SDS-PAGE, followed by autoradiography. $T4_{ex1}$ protein (protein encoded by the modified T4 DNA) produced in the Cos system can be readily co-precipitated with anti-gp120 antibody in the presence of gp120. Rabbit heteroantisera to the CD4 protein ($T4_{ex1}$) is also available for identification of modified CD4 products in which monoclonal CD4 epitopes are no longer present. Thus, this makes it possible to be certain that CD4-related protein is being translated in Cos even in the absence of gp120 binding material. Co-precipitation of gp120 with $T4_{ex1}$ protein produced in Cos is readily detected in the presence of anti-gp120 antibody plus rabbit anti-mouse Ig. The co-precipitated product will be a 50KD band in SDS-PAGE analysis after autoradiography. The fact that no equivalent ^{35}S-cysteine labelled $T4_{ex1}$ band is detected in the absence of gp120 demonstrates the specificity of this reaction.

The modified soluble CD4 protein produced as described above includes the amino acid sequence (as shown in Figure 1) of the CD4 protein through amino acid 183. In addition to its ability to bind to HIV and, thus, interfere with infection of cells by the virus, this truncated soluble CD4 protein has the further advantage that it lacks the glycosylation sites present in $T4_{ex1}$ and should, thus, be less immunogenic. In addition, the terminal amino acid (histidine) present in the mature CD4 protein encoded by the nucleotide sequence of Figure 1 is absent from the biologically active, modified soluble CD4 fragment of the present invention. It is also absent in the native CD4 molecule. Because the

glycosylation sites are not present in the CD4 fragment produced in this manner, fragments of this type can be expressed in a bacterial host. Immunoprecipitation of CD4 protein having amino acid residues 1-182 (as represented in Figure 1) identifies a band of approximately 19 kD on SDS-PAGE of 19Thy5D7 immunoprecipitates from transfected Cos supernatants. Co-precipitation studies with gp120 and anti-gp120 antibody identifies the same band.

As explained previously, there are many possible useful modifications (e.g., changes in amino acid sequence, truncation) of CD4 protein which can be produced as described above for truncation of the protein at amino acid 183. Some of these modifications have been described above. Additional modifications can be made at other sites within the CD4-encoding DNA, with the outcome that expression of the modified DNA will result in production of modified soluble human CD4 fragments. Biological activity (e.g., ability to bind HIV gp120 and interfere with HIV infection of cells) can be assessed as described herein.

For example, truncation at a different amino acid can be carried out. In one case, truncation of $T4_{ex1}$ after amino acid 369 (i.e., removal of the carboxy terminal histidine) is carried out in a similar manner, by insertion of a termination codon (see Figure 1). It is reasonable to expect that the resulting truncated form will retain the capability of binding HIV. In addition, such a modified form has the advantage that it lacks the histidine present in the CD4 protein encoded by the cDNA of Figure 1 and not present in the native molecule.

Another approach is to produce biologically active, modified soluble CD4 fragments in such a manner that fragments which include of one or more domains of the encoded protein are obtained.

Production of fragments of the present invention in which one or more of the domains is present is of interest, for example, because of the importance of at least the first two domains (see Figure 7) in binding of CD4 with HIV. That is, it is known that the external segment of CD4 (T4) functions as the T cell surface receptor for HIV, by binding the major HIV coat protein (gp120) with relatively high affinity.

However, the region of the CD4 molecule that binds gp120 has not yet been defined. Nor is it known whether the same or different segments of CD4 bind to an invariant region of class II MHC molecules(s) which are the presumed physiologic CD4 ligand. Meuer, S. et al., Proceedings of the National Academy of Sciences, USA, 79:4395 (1982); Biddison, W. et al., Journal of Experimental Medicine, 156:1065 (1982); Gay, D. et al., Nature, 328:626 (1987). In this regard, sequence analysis of CD4 has suggested an evolutionary origin from a structure with four immunoglobulin-related domains (Figure 7A). Two of these domains (the first two) are involved in HIV gp120 binding. The NH_2-terminal CD4 domain (amino acids 1-92), termed domain 1, bears the most structural homology to Ig light chain variable regions (about 32% at amino acid level). Eight of 14 invariant residues are conserved between domain 1 of T4 and V_{Kappa} domains. Maddon, P. et al., Cell, 42:93 (1985). The first and second

cysteines (amino acids 16 and 84; Figure 7A and 7B) in domain 1 of the CD4 sequence are separated by 67 amino acids, which are positions and spacings similar to members of the Ig family. By analogy to sheep and mouse CD4, these cysteines in human CD4 also form a conserved intrachain disulfide bond characteristic of V domains. In addition, secondary structural analysis suggests the presence of seven beta strands within the CD4 domain 1. Cysteines bridged by intrachain disulfides form the boundaries of hypothetical domains 2 (amino acids 120 and 151) and 4 (amino acids 303 and 345), with certain short stretches of Ig-like sequences clustered around them. In contrast, no cysteines are found in domain 3, although the latter bears homology by sequence alignment with a poly Ig receptor.

The NH_2-terminal region of CD4, including the immunoglobulin V-like domain, has been shown to be required for gp120 interaction. In contrast, the carboxy terminal half of the molecule containing the two potential N-glycosylation sites does not appear to be necessary.

For example, insertion of a TAA termination codon after the valine codon at position 128(GTG, Figure 1) will result in production of a domain 1 $T4_{ex1}$ construct. A domain 1 $T4_{ex1}$ construct and a partial domain 2 mutant will be obtained if a TAG termination codon is inserted after the fourth cysteine, which produces truncation after amino acid 162 (Figure 1). This will produce a modified soluble CD4 fragment capable of binding HIV and will also make manufacture/production easier because of

improved ability to introduce the construct into a cell line.

In a similar manner, production of a domain 1, 2 and partial domain 3 construct can be carried out. In this case, the triplet encoding the glutamine at amino acid position 243 (see Figure 1) will be altered to a TAG termination codon. This will result in production of a modified soluble CD4 protein having the same advantages described above for the protein resulting from termination after the fourth cysteine.

The method described herein can be used, with appropriate modification, to convert asparagine and N-linked glycosylation sites at positions 271 and 300 to asparate. This can be carried out in the same construct or in two separate constructs (each including one of the two modifications). In either case, the two codons at the positions indicated will be modified: in the case of the codon for amino acid 271, to GAC and in the case of the codon for amino acid 300, to GAT. This modified protein will also bind HIV and has the further advantage that because the glycosylation sites are no longer present, it will be less immunogenic than a fragment which includes such sites.

Additional modification of the mature CD4 protein can similarly be made, as desired, and subsequently shown to have the capability of binding HIV by the means described herein. Expression of additional constructs (DNA encoding additional modified soluble CD4 fragments) will be carried out, for example, in baculovirus (e.g., Autographa

californica), Chinese hamster ovary (CHO) cells or E. coli.

In the case of production in baculovirus, this will be carried out as follows and in a similar manner to that described by Hussey et al. and Smith et al., the teachings of which are incorporated herein by reference. Hussey, R.E. et al., Nature, 331: 78-81 (1988); Smith G., et al., Proceedings of the National Academy of Sciences, USA, 82: 8404-8408 (1985).

Transfer of the $T4_{ex1}$ sequence from the plasmid vector to the Autographa californica nuclear polyhedrosis virus (AcNPV) genome can be accomplished essentially as described in Smith et al., (1985) Proceedings of the National Academy of Sciences, USA, 82:8404-8408. Cotransfection by calcium phosphate precipitation of 4 ug pAc373/$T4_{exi}$ DNA with 1 ug of purified AcNPV DNA into Spodoptera frugiperda (SF9) cells, which are publicly available, results in homologous recombination between the recombinant sequence of the transfer vector and the polyhedrin gene sequence of AcNPV. Recombinant AcNPV contains an inactivated polyhedrin gene which no longer forms occlusions in infected cells, thus providing a means by which infected and noninfected cells can be distinguished. For plaque purification, 2 x 10^6 SF9 cells can be seeded in 100 mM petri dishes 24 hours prior to assay. Ten fold dilutions of viral supernatant are prepared, using final media (Grace's insect medium (Gibco, Grand Island, NY), TC yeastolate 0.33%, lactalbumin hydrosylate 0.33%, 2 mM supplemental glutamine and 50 ug/ml gentamycin containing 10% FCS (Hyclone, Logan, UT). Each plate

is innoculated with virus (e.g., 1 ml., 10^{-3} to 10^{-7} dilution) plus 2 ml of final media. After incubation for 2 hours, the innoculum is removed and replaced with 10 ml of 1.5% Sea Plaque agarose (FMC Bioproducts, Rockland, ME) in final media. Plates are transferred to a a humid environment after agarose solidification for 4-6 days at 27°C.

Plaque assay of the transfection supernatant will demonstrate distinct morphological differences between infected cells; infected cells which are occlusion positive contain wild type AcNPV and infected cells which are occlusion negative contain recombinant CD4 virus. Occlusion-negative plaques are identified, selected, and further plaque purified. DNA from cells infected with putative CD4 recombinant virus will be hybridized with a ^{32}P labelled CD4 cDNA probe to verify the presence of the CD4 sequence.

Production of the $T4_{ex}$ polypeptide is carried out as follows: SF9 cells (6×10^5 cells per well) are seeded per well in 24 well Nunc plates (Interlab, Thousand Oaks, CA) for 2 hours at 27°C and then adherent SF9 cells are infected with virus at an MOI of 10 in 0.2 ml final media for 2 hours. The innoculum is then removed and cells are cultured in 0.5 ml fresh medium at 27°C for 48 hours. Adherent cells are then washed twice with 0.5 ml Grace's medium lacking serum and methionine. This is followed by incubation in 0.5 ml in the same medium for 1 hour. The adherent cells are washed once and then cultured for 6 hours in serum and methionine-free Grace's medium containing 67 uCi ^{35}S methionine (New England Nuclear, Boston, MA 1134

Ci/nmol). Culture supernatants are harvested, microfuged for 10 minutes, and dialyzed at 4°C against PBS containing 0.5% sodium azide and 10 mM cold methionine. Cells are dislodged from the wells, washed twice with Grace's medium at 4°C (by centrifugation in a Sorvall RT6000 for 5 min at 1000 rpm) and finally lysed for 30 min at 4°C by the addition of a RIPA buffer containing 1% Triton X-100, 0.15 M NaCl and a cocktail of protease inhibitors, as described below. The lysates are microfuged for 10 min and dialyzed at 4°C using the same procedure as was used for culture supernatants.

Both lysates and culture supernatants are subjected to immunoprecipitation for 16 h at 4°C with a monoclonal anti-CD4 antibody (19Thy5D7) linked to Affigel-10 beads (5 mg monoclonal antibody/ml gel). After immunoabsorption, the beads are washed twice with lysis buffer and bound material is eluted by treatment of the beads with 0.1M glycine-HCl buffer, pH 2.0. Eluates and whole samples of lysates or culture supernatants are mixed with SDS sample buffer containing 2-mercaptoethanol, boiled for 5 minutes and electrophoresed in 12.5% mini-slab gels according to Laemmli. Laemmli, Nature, 227:680-685 (1970). Subsequently, the gels are fixed, dried and autoradiographed using Kodak XAR-5 film.

High titer viral stocks are generated by infecting SF9 cells at an MOI of 1 and culturing at 1 x 10^6 cells/ml for 4 days in final media. These stocks are used for infecting SF9 cells for production of protein. For large scale production of protein, SF9 cells are grown in 2 liter spinner flasks in final media. Cells are harvested and

infected with an MOI of 15 (using high titer viral stocks) at a concentration of 10 x 10^6 cells/ml.

Cells are then pelleted, resuspended in media at 1 x 10^6/ml, and cultured for 3 days at 27°C in spinner flasks. At this time, supernatants are collected by centrifuging cultures to remove cells.

For large scale purification, infected SF9 cell culture supernatants are harvested by centrifugation of cells in a Sorvall H-4000 rotor at 800 rpm for 6 minutes at 4°C. The culture supernatants are then subjected to protease inhibition by the addition of a cocktail of protease inhibitors made up of leupeptin, antipain, pepstatin, and chymostatin to final concentrations of 0.5 ug/ml; soybean trypsin inhibitor to 0.02 ug/ml; and phenyl methyl sulfonyl fluoride (PMSF) to 1.25 mM, followed by adjustment of the pH to 6.8 by the dropwise addition of 1 M NaOH. The samples are subsequently clarified by centrifugation in a Sorvall GSA rotor at 8000 rpm for 25 minutes at 4°C and pumped at 4°C at a flow-rate of 30 ml/hour through a 2 ml precleared immunoabsorbent column, 21Thy2D3 monoclonal antibody (anti-T8) coupled to Affigel-10 (Biorad), followed in series by a 7 ml column of anti-CD4 monoclonal antibody (19Thy5D7) coupled to Affigel 10 at a concentration of 7.5 mg monoclonal antibody per ml of gel. The monoclonal antibodies are made according to conventional methods. The anti-CD4 column is then washed with 30 ml of 10 mM Tris-HCl buffer, pH 6.8 followed by 15 ml of 0.1M glycine-HCl, pH 5.0. The bound CD4 polypeptides are eluted by pumping 0.1 M glycine-HCl, pH 2.0 through the washed anti-CD4 column and 0.8 ml fractions of eluant are collected

into tubes containing 0.15 ml 1 M Tris-HCl, pH 7.5. During the whole column fractionation procedure, eluate absorption is monitored at 280 nm with a Uvicord 2 (LKB, Gaithersburg, MD) fitted with an event marker. Fractions of neutralized pH 2.0 eluate containing protein are pooled and concentrated by ultrafiltration in a stirred cell (Amicon, model 3) fitted with a YM-5 membrane. Typically the yield of purified $T4_{ex}$ polypeptides is approximately 1 ug/ml of infected SF9 culture supernatants. Aliquots containing 1 ug of protein concentrate (assuming that 1 OD unit = 1 mg/ml at a 280 nm) are examined for purity in 12.5% SDS-polyacrylamide slab gels, followed by staining with Coomassie blue.

Polypeptides produced in this manner, can be purified and characterized using known methods, to confirm that they are, in fact, those encoded by the modified CD4 cDNA introduced into the cells as described.

Alteration of Ability of CD4 Fragments to Bind HIV gp120

Regions or sites on human CD4 critical for HIV gp120 binding were identified, as described below and, based on the identification of critical sites, soluble human CD4 fragments with altered HIV gp120 binding ability were produced, as is described below, particularly in Examples 4 and 5.

Identification of Amino Acid Residues of Human CD4 Critical for gp120 binding

The extracellular segment of murine CD4 is overall 50% identical to its human counterpart

(Maddon, P.J., et al., Proc. Natl. Acad. Sci. USA, 84: 9155-9159 (1987) at the amino acid (a.a.) level but fails to bind gp120. McClure, M.O., et al., Nature, 330: 487-489 (1987) These differences were used in precisely defining those residues of human CD4 critical for gp120 binding. Substitutions of all non-conserved murine for human CD4 residues between amino acid positions 27-167 were made. To this end, oligonucleotide-directed mutagenesis was used to create each of 16 individual mutant human CD4 molecules containing from 1 to 4 amino acid substitutions. Introduction of as few as three amino acids into corresponding positions of human CD4 resulted in CD4 fragments unable to bind gp120. These critical residues have been shown to be located in domain I as well as in domain II of CD4, thus implying that the gp120 binding site is complex and involves both of the NH_2-terminal domains. Modelling studies using the 3-dimensional coordinates of the V_K Bence-Jones homodimer, REI, localize the site of domain I to the C'' β strand. Thus, domain I is distant from the loops analogous to hypervariable regions.

Residues of the CD4 structure involved in HIV gp120 binding were characterized through use of a Cos-1 cell expression system and a cDNA encoding the anchor-minus CD4 segment termed $T4_{ex1}$. Hussey, R.E., et al., Nature, 331: 78-81 (1988) The 370 amino acid $T4_{ex1}$ protein (Figure 1) contains 369 of the predicted 372 NH_2-terminal amino acids of the CD4 extracellular segment and a COOH-terminal histidine. As shown in Figure 8, this structure is comprised of three intrachain disulfide bonded domains (a domain

is defined as residues between and including 20 amino acid residues to either side of the cysteines), and one domain (III) which lacks cysteine residues but, like its counterparts, is immunoglobulin-like. Clark, S., et al., Proc. Natl. Acad. Sci. USA, 84: 1649-1653 (1987). Nanomolar concentrations of $T4_{ex1}$ inhibit gp120-transmembrane CD4 interaction, syncytium formation and HIV infection by binding to gp120-expressing cells. Hussey, R.E., et al., Nature, 331: 78-81 (1988).

As described in Example 4, the $T4_{ex1}$ construct was subcloned into the vector CDM8 and transfected into Cos-1 cells. Seed, B., et al., Proc. Natl. Acad. Sci. USA, 84: 3365-3369 (1987) Supernatants from metabolically labelled transfected cells were tested by immunoprecipitation with an anti-CD4 monoclonal antibody (19Thy5D7). The resulting precipitate was subjected to SDS-PAGE. Results showed the presence of a 50KD CD4-derived molecule in transfected Cos-1 cell supernatants (Figure 9, lane 3). The same molecule is co-precipitated from Cos-1 supernatants with an anti-gp120 monoclonal antibody after preincubation of the supernatant with gp120 (Figure 9, lane 5). These reactions are specific for $T4_{ex1}$, as demonstrated by the fact that 1) an irrelevant antibody (anti-T6) fails to precipitate $T4_{ex1}$ (Figure 9, lane 1) and 2) no CD4 band is detected with anti-gp120 antibody in the absence of gp120 (Figure 9, lane 6).

Prior studies, described above, employing either CD4 DNA truncation or proteolytic digestion demonstrated that the residues critical for gp120 interaction reside in domains I and/or II

exclusively. Traunecker, A., et al., Nature, 331: 84-86 (1988); Berger, E.A., et al., Proc. Natl. Acad. Sci. USA, 85: 2357-2361 (1988) (Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA, in press) Similarly, the Cos-1 cell derived product of a $T4_{ex1}$ protein truncated after amino acid residue 182 (by insertion of a stop codon in the cDNA sequence) is precipitated as a 20KD protein by anti-CD4 antibody and binds to gp120 (Figure 9, lanes 4 and 7, respectively). In contrast, expression of a cDNA truncated at amino acid 110 (containing domain I only) failed to give rise to a gp120 binding protein. (Example 4) These data suggest that both domains I and II are required for HIV gp120 binding.

Therefore, further analysis of the CD4-gp120 interaction was carried out by creating 35 amino acid substitutions which encompass all non-conservative mouse-human species differences within the first two domains of CD4 between amino acid residues 25 and 167. The NH_2-terminal CD4 amino acids were not considered here because an NH_2-terminal synthetic peptide failed to block HIV gp120 binding, even at millimolar concentrations. For each substitution, an amino acid of the human sequence was replaced with the amino acid found in the equivalent position of the murine CD4 sequence. Maddon, P.J., et al., Proc. Natl. Acad. Sci. USA, 84: 9155-9159 (1987). The murine CD4 sequence does not bind gp120, and, thus, it was anticipated that some murine substitutions would abrogate human CD4-gp120 interaction. As shown in Table 1, 15 oligonucleotides were used in a standard site-directed mutagenesis protocol, as described in Example 4, to produce 16 different

versions of the human CD4 molecule containing from 104 substitutions each. The positions of these substitutions are listed in Table 1 and diagramatically mapped in Figure 8. All 16 CD4 mutants were assayed after transfection into Cos-1 cells by immunoprecipitation with anti-CD4 monoclonal antibody and by gp120 co-precipitation with anti-gp120.

TABLE 1. PRODUCTION AND ANALYSIS OF CD4 SITE-DIRECTED MUTANTS

Mutant	Oligonucleotide used for mutagenesis	Amino acid change	Anti-CD4 immuno-precipitation	Anti-gp120 co precipitation
M1A	Mouse subst. T F D I[a] 223 CAA-TTC-ACC-TGG-AAA-TTC-TCC-GAC-CAG-AGA-AAC 255 Human aa N N N I	aa 27 N to T aa 30 N to F aa 32 N to D	+	+
M1B	T F D R 223 CAA-TTC-ACC-TGG-AAA-TTC-TCC-GAC-CAG-AGA-AAC 255 N N N I	aa 27 N to T aa 30 N to F aa 32 N to D aa 34 I to R	+	+
M2	H 261 G-CGA-AAT-CAC-GGC-TCC 276 Q	aa 60 Q to H	+	+
M3	G P S 283 ACT-AAA-GGT-GGA-TCC-CCG-AGT-AAT-GAT-CG 311 P K L	aa 48 P to G aa 50 K to P aa 51 L to S	+	-
M4	K 335 GG-GAC-AAA-GGA-AAC-TTC 351 Q	aa 64 Q to K	+	+
M5	N K 355 CTG-ATC-ATC-AAT-AAG-CTT-AAG 375 K N	aa 72 K to N aa 73 N to K	+	+
M6	Q 382 GAC-TCA-CAG-ACT-TAC-ATC 399 D	aa 80 D to Q	+	+
M7	N R E 406 CTG-GAG-AAC-CGG-AAG-GAG-GAG-GTG-GAA-TTG-C 436 D Q Q	aa 88 D to N aa 89 Q to R aa 94 Q to E	+	+
M8	K P S 436 CTA-GTG-TTC-AAA-TTG-ACT-GCC-AAC-CCT-GAC-ACC-AGC-CTG-CTT-C 478 G S N	aa 99 G to K aa 104 S to P aa 107 N to S	+	+
M9	S K V 499 ACC-TTG-GAC-AGC-AGC-AAG-GTT-AGT-AGC-CCC 528 P P G	aa 121 P to S aa 122 P to K aa 123 G to V	+	-[c]
M10	L T E 520 ACT-AGC-CCC-CTA-ACG-GAA-TGT-AGG 543 S V Q	aa 127 S to L aa 128 V to T aa 129 Q to E	+	+
M11	H K V 534 G-CAA-TGT-AGG-CAT-AAA-AGG-GGT-AAA-GTC-ATA-CAG-GC 569 S P N	aa 132 S to H aa 133 P to K aa 137 N to V	+	+
M12	V 570 G-GCG-AAG-GTC-CTC-TCC-G 586 T	aa 143 T to V	+	+
M13	R 590 CT-CAG-CTG-CGG-CTC-CAG-G 607 E	aa 150 E to R	+	+
M14	D F N 606 G-GAT-AGT-GAC-TTC-TGG-AAT-TGC-ACT-GTC 633 G T T	aa 155 G to D aa 156 T to F aa 158 T to N	-[b]	-[c]
M15	T L D 626 CC-ACT-GTC-ACG-CTG-GAC-CAG-AAC 648 L Q N	aa 162 L to T aa 163 Q to L aa 164 N to D	+	+

[a] Two mutants were recovered from the mutagenesis using this oligonucleotide; one contained mutations at amino acid 27, 30 and 32 but not 34 and the second contained all four changes. These two mutants were transfected separately.

[b] M14 was also negative when tested for immunoprecipitation with anti-CD4 monoclonal OKT4A

[c] A very faint 50KD band (~10 fold less intense than $T4_{exl}$) was observed upon coprecipitation with gp120.

Mutagenesis, immunoprecipitation and coprecipitation procedures are described in the legend to Fig. 1.

Immunoprecipitation of the original $T4_{ex1}$ and four representative mutants is shown in Figure 10 (panel a). In addition to $T4_{ex1}$, each of the mutants M5, M10, M7 and M3 react with the anti-CD4 monoclonal antibody 19Thy5D7. As shown in Table 1, 15 of the 16 mutants react with anti-CD4 antibody. Only mutant M14 did not react; it was also unreactive with OKT4A, which is a second monoclonal antibody directed at a different CD4 epitope.

Thirteen of the 16 mutants bound gp120 in a manner equivalent to $T4_{ex1}$, as judged by the co-precipitation assay. Figure 10 (panel b) demonstrates that $T4_{ex1}$, M5, M10 and M7 are all co-precipitated by anti-gp120 in the presence of gp120. Overall, a 2-3 fold experimental variation in co-precipitation with gp120 was observed ($T4_{ex1}$ vs. M5 in panel b Figure 10). Among gp120-binding CD4 proteins, however, a positive signal was observed in every experiment (using a minimum of 2-3 separate transfections). In contrast, although M3 is recognized by anti-CD4 antibody, it fails to bind to gp120 (Figure 10, panel b). In addition, M9 (Table 1) has a substantially reduced gp120 binding capacity, although anti-CD4 monoclonal antibody immunoprecipitates a band of identical size and intensity to $T4_{ex1}$. M3 contains three amino acid substitutions in human CD4 domain I at positions 48, 50 and 51. One or more of these changes clearly abrogates the ability of CD4 to bind to HIV gp120. M9 contains three amino acid substitutions in domain II of CD4 at positions 121-123. Thus, alteration of a few residues in either CD4 domain I or domain II results in abrogation of HIV gp120 binding.

In addition, M14 demonstrates reduced binding to gp120 (Table 1). M14 also failed to bind to the two anti-CD4 monoclonal antibodies examined. Thus, one cannot rule out the possibility that the three substitutions in M14 (at positions 155, 156 and 158) somehow decrease the expression of this mutant CD4 protein. It is more likely that these substitutions have destroyed both the gp120 binding site and the epitopes recognized by the two monoclonal antibodies, perhaps through a general disruption of the CD4 protein's 3-dimensional structure because translation of in vitro transcribed RNA from M14 gave results identical to $T4_{ex1}$ transcribed RNA.

The contribution of CD4 domain 1 to gp120 binding was recognized previously in studies of the $T4_{ex1}$ polypeptide produced in a baculovirus system in conjunction with proteolytic fragmentation analysis, microsequencing and a specific CD4-gp120 binding assay. Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA. Richardson and co-workers showed that disruption of the peptide bond at lysine 72 by tryptic cleavage destroyed CD4-gp120 interaction without inducing any detectable alterations in other domains of CD4. Furthermore, reduction of intrachain disulfide bonds in the CD4 molecule also abrogated high affinity gp120 binding, thereby strongly implying that the binding site for gp120 is complex and depends on the stabilized CD4 structure. Whether the domain I and II mutations introduced in the work described herein affect gp120 contact residues themselves or, alternatively, affect the tertiary structure around the contact residues cannot be concluded at present. Footprint analysis of

CD4-gp120 protein-protein interactions or analysis of CD4-gp120 cocrystals will be necessary to determine the effect of the mutations described. Nevertheless, the ability of a synthetic peptide comprising amino acid residues 23-56 to inhibit syncytium formation at 10^{-4} M may support the notion that residues 48, 50 and/or 51 contribute to the gp120 binding sites. Jameson, B.A., et al., Science, 240: 1335-1339 (1988)

Eight residues are conserved between domain I of CD4 and the 14 invariant residues of the Kappa light chain variable (V) regions. Maddon, P., et al., Cell, 42: 93-104 (1985). In addition, the first and second cysteines (amino acids 16 and 84) in domain I of CD4 are separated by 67 amino acids, positions and spacing similar to those of members of the immunoglobulin family. Furthermore, secondary structural prediction suggests the presence of eight Kappa strands in CD4 domain I. In light of these homologies to Ig, CD4 domain I was modelled on the basis of the known 3-dimensional coordinates of the V_k Bence-Jones homodimer, REI. Use of this model has resulted in accurate prediction of each of three tryptic cleavage sites in domain I to be surface exposed, thus supporting the validity of the CD4 model. Richardson, N.E., et al., Proc. Natl. Acad. Sci. USA. It was therefore of interest to determine the relative positions of the M3 mutations at amino acid residues 48, 50 and 51 of CD4.

The region of residues in the alpha carbon skeleton of the REI homodimer corresponding to the mutated CD4 residues which abrogate gp120 binding were determined. This region corresponds to the C'' strand unique to V domains which connect the two

sheets. Williams, A.F., et al., Ann. Rev. Immunol., 6, 381-405 (1988). The alignment between REI and CD4 requires a gap in this segment, and, thus, it is not meant to imply that the CD4 alpha carbon skeleton follows an identical course in this region. Nevertheless, it is very likely that the CD4 sequence will loop out and be solvent exposed. Furthermore, it should be noted that this site is distinct from the three segments equivalent to the hyper-variable loops of the REI homodimer.

Based on the above analysis, one prediction would be that if gp120 does contact residues in the region analogous to the C'' strand of REI, it might also contact residues in CD4 domain II adjacent to this region. Perhaps M9 and/or M14 mutations are localized to such sites. That domains I and II of CD4 might be spatially close to one another in some regions is further supported by antibody competition studies in which an antibody (OKT4A) whose epitope was mapped to a region in domain I showed reciprocal competitive binding with two antibodies (OKT4F and OKT4B) whose epitopes mapped to domain II. Jameson, B.A., et al., Science, 240: 1335-1339 (1988).

The region of CD4 domain I implicated as a possible binding site for gp120 is distinct from the loops analagous to hypervariable complementarity determining segments. If those loops form a binding site for class II MHC, the putative natural ligand of CD4 one can speculate that gp120 may be incapable of inhibiting class II recognition events, even after binding to the CD4 structure. Krensky, A.M., et al., Proc. Natl. Acad. Sci. USA, 79: 2365-2369 (1982); Meuer, S.C., et al., Proc. Natl. Acad. Sci. USA, 79:

4395-4399 (1982); Biddison, W., et al., J. Exp. Med., 156: 1064-1076 (1982); Marrach, P., et al., J. Exp. Med., 158: 1077-1091 (1983); Doyle C., et al., Nature, 330: 256-259 (1987) The CD4 mutants described herein should be useful in future analysis of CD 4- class II MHC interactions.

As a result of the identification of sites critical to binding of CD4 to the HIV gp120 envelope protein, it is now possible to produce modified soluble human CD4 fragments whose ability to bind gp120 is altered (i.e., whose ability to bind gp120 is different from that of the corresponding naturally-occurring human CD4 fragment). As described in the previous sections and in Examples 4 and 5, such sites have been identified by oligonucleotide-directed mutagenesis used to create 16 mutant human CD4 molecules which resulted in substitution of all non-conserved murine amino acid residues for human CD4 residues between amino acid positions 27-167, as represented in Figure 1.

As shown in Table 1, 15 of the 16 CD4 "mutants" created as described react with anti-CD4 monoclonal antibody 19thy5D7 and 13 of the 16 bind gp120 in a manner equivalent to the gp120 binding evidenced by $T4_{ex1}$. Three mutants, designated M3, M9 and M14, do not exhibit gp120 binding equivalent to that of $T4_{ex1}$: M3 fails to bind gp120; M9 has substantially reduced gp120 binding capacity; and M14 demonstrates reduced gp120 binding ability. As also shown in Table 1, M3 and M9 are recognized by anti-CD4 antibody and M14 is not recognized by either of the two anti-CD4 antibodies used.

These results demonstrate that these sites are critical for gp120 binding by CD4 and that the changes made in the amino acid sequence of human CD4 (as represented in Figure 1) to produce these CD4 mutants resulted in elimination of or reduction in gp120 binding. In a similar manner, other changes at one or more of these critical sites can result in elimination of or reduction in gp120 binding ability. Conversely, amino acid residues can be introduced at these critical sites to produce modified soluble human CD4 fragments with enhanced gp120 binding ability.

Such substitutions can be made: 1) at one, two or all three of the critical sites (i.e., at one or more of the three amino acid sites represented by mutants M3, M9 and M14) and/or 2) of one, two or all three amino acid residues within each site (i.e., within a critical site, of amino acid residues 1, 2 or 3 individually, 1, 2 and 3 in any combination of a 2 amino acid residues; or of all three amino acid residues).

For example, in mutant M3, glycine, proline and serine, respectively, replace proline, lysine and leucine, which occur at amino acid positions 48, 50 and 51 of human CD4. Substitution of one or more of those amino acids by other amino acids of the same type (e..g, at position 48 by another amino acid with a nonpolar R group) as that present at that position in M3 can be made and the effect on gp120 binding ability determined.

Substitutions at these three sites, individually or in combination, of amino acids having characteristics different from those of amino acid

whose presence at those sites has been shown to eliminate or reduce gp120 binding ability can also be made and their effect on binding ability assessed using the anti-CD4 immunoprecipitation and anti-gp120 coprecipitation methods described in the Examples. In particular, substitutions of some or all of the amino acids at one or more of these critical sites which result in modified soluble CD4 fragments with enhanced gp120 binding ability can be made. Using the techniques described herein, CD4 fragments having enhanced binding ability can be identified.

One approach to producing modified soluble human CD4 fragments with enhanced gp120 binding ability is as follows: amino acid residues present at the three sites in human CD4 (as represented in Figure 1) and amino acid residues present at the corresponding positions in the three mutant CD4 molecules are excluded from the group of amino acid residues to be assessed for their effects on gp120 binding ability when they are incorporated at these sites. Also excluded are amino acids having similar characteristics (e.g., nonpolar R groups, uncharged polar R groups, etc.). Mutants are then produced to include amino acid residues other than those eliminated from consideration in this manner. Each mutant is then assessed using the anti-CD4 immunoprecipitation and anti-gp120 coprecipitation techniques described.

As a result, modified soluble human CD4 fragments having enhanced gp120 binding ability can be identified. Similar techniques can be used to identify additional critical sites, if such sites exist, and, subsequently, to make substitutions and

assess their effects on gp120 binding ability of the resulting modified soluble CD4 fragments.

Production of Modified Soluble CD4 Fragments Having Altered gp120 Binding Ability

Modified soluble CD4 fragments having altered gp120 binding ability are produced using the techniques described in detail in Examples 4 and 5. Briefly, they are produced as follows:

DNA encoding a desired CD4 fragment is produced, either by using recombinant DNA techniques, such as excising it from a vector containing cDNA encoding such a fragment, or by synthesizing DNA encoding the desired fragment mechanically and/or chemically, using known techniques. DNA produced by these techniques encodes a soluble CD4 fragment which includes none of the hydrophobic transmembrane region of CD4 or a portion of that region (generally six amino acids or less) small enough that it does not prevent solubilization of the fragment. In addition, particularly in the case of CD4 fragments having enhanced gp120 binding ability, the CD4 fragment is long enough (e.g., 10 amino acids or more) to bind effectively to HIV gp120 envelope protein.

Templates for subsequent mutagenesis are produced, using the CD4 fragment-encoding cDNA or DNA. As described below, this can be carried out using a single-stranded bacteriophage cloning vehicle, such as M13. This results in production of single-stranded DNA homologous to only one of the two strands of the DNA encoding the desired CD4 fragment. The resulting single-stranded DNA is used as a

template for producing the desired modified soluble CD4 fragments, as follows:

Oligonucleotides are produced, such that their sequence includes a base change or changes which, when incorporated into the nucleotide sequence of DNA subsequently used for the production of CD4 fragments, results in the desired change in the encoded CD4 protein (i.e., different from that encoded by the nucleotide sequence of Figure 1). Such oligonucleotides are produced using standard methods. Oligonucleotides having a base change or base changes are referred to as mutagenized or mutant oligonucleotides.

The mutant oligonucleotide produced in this manner is hybridized to (e.g., by being kinased) the template produced as described above, to produce a template-mutant oligonucleotide complex, referred to as a mutant primer/template. The mutant primer/template is used for the production of a second strand of DNA, using well-known techniques. For example, synthesis of the second DNA strand is carried out by the Klenow fragmnet of DNA polymerase in the presence of dCTPαS. Taylor, J.W. et al., Nucleic Acids Research, 13:8749-8764 (1985); Taylor, J.W. et al., Nucleic Acids Research, 13:8764-8785 (1985); Nakayame, K. and F. Eckstein, Nucleic Acids Research, 14:9679-9698 (1986). The resulting strand of DNA contains a modification (or modifications) in the nucleotide sequence of T4 cDNA (i.e., is different from the nucleotide sequence represented in Figure 1) and is referred to as a mutant strand.

Unreplicated single-stranded DNA is removed and the double-stranded DNA is nicked with a selected

restriction enzyme (e.g., NciI, which does not cut phosphorothioate DNA and, thus, does not cut the new DNA strand containing dCTP S or the mutant strand). Nicked, nonmodified DNA is removed by digestion with another enzyme, such as exonuclease III. The resulting gapped DNA is repolymerized and, because the mutant strand serves as the template for repolymerization, the mutation or modification is copied into both strands.

Once produced, the double-stranded DNA, in which both strands contain the mutation or modification encoding the corresponding modification in the amino acid sequence of the desired soluble CD4 fragment is introduced into a competent host cell, such as a competent bacterial host (e.g., by transformation). The resulting plaques are grown and DNA contained in them is isolated, using known techniques, and sequenced to confirm the presence of the mutation.

The mutated DNA produced in this manner is excised from the M13 vector containing it, introduced into a suitable expression vector, such as CDM8, and transfected into an appropriate host cell, such as Cos cells, in which it is expressed. Aruffo, A. and B. Seed, Proceedings of the National Academy of Sciences, USA, 84:3365-3369 (1987). As a result, mutant CD4 proteins can be assayed, using known techniques. The vector-insert ligation mixture is introduced into competent host bacteria, such as the publicly available E. coli MC1061P3, and radiolabelled T4 DNA is used to identify CDM8 containing mutant T4 cDNAs.

Production, in Cos cells transfected with the vector containing mutant T4 cDNA, of modified soluble

CD4 fragments having the desired alteration in gp120 binding ability is subsequently assayed, using known techniques described below.

As a result of this procedure, double stranded DNA encoding a modified soluble CD4 fragment having altered gp120 binding ability is produced, the encoded CD4 fragment is expressed and its ability to bind the HIV gp120 envelope protein is assessed.

An alternative approach to producing modified soluble human CD4 fragment having altered gp120 binding ability is to use peptide synthesis to make a peptide or polypeptide having the amino acid sequence of such a fragment.

The above-described technique was used for producing the 16 mutant CD4 fragments whose sequences are represented in Table 1. Construction of the 16 mutants, transfections, immunoprecipitations and co-precipitations were carried out as described in Example 4. The presence of each mutant was confirmed by directly sequencing the plasmid DNA used for individual transfections.

Use of Soluble Human CD4 Fragments

Soluble human CD4 fragments of the present invention have diagnostic, preventative and therapeutic applications. For example, biologically active soluble human CD4 fragments can be used for diagnosis, therapy and prevention of infection by HIV.

For example, such fragments can be used therapeutically (in vivo) to treat individuals infected with HIV. Such fragments can be administered by an

acceptable route (e.g., intravenously, intramuscularly, intraperitoneally, orally), alone or after combination with an acceptable carrier (e.g., saline buffer). They can be administered to inhibit binding of HIV to T4 lymphocytes and to inhibit HIV transmission from an infected cell to uninfected cells by interfering with syncytium formation. The quantity of such CD4 fragments administered will be determined on an individual basis, but will generally range from approximately 10 ug/kg body weight to approximately 500 ug/kg body weight per day (in one or more doses per day).

Biologically active soluble CD4 fragments of the present invention can also be used for diagnostic purposes. For example, they can be used in known immunoassay procedures for detecting the presence and determining the quantity, if desired, of HIV gp120 envelope protein (and, as a result, of HIV itself) in samples, such as blood, semen and saliva. CD4 fragments of the present invention can be, for example, attached or bound by virtue of the CD4 fragment to a solid support, such as latex beads, which are then contacted with a sample to be assayed, in such a manner that if HIV is present in the sample, it will be bound (by virtue of the CD4 frament-gp120 interaction). This can be followed by precipitation and/or labelling through contact with an anti-gp120 antibody and detection of the precipitate or labelled product, using known techniques.

Biologically active soluble CD4 fragments can also be used for the prevention of HIV infection. For example, such fragments can be incorporated in or attached to materials which might come in contact

with HIV. They can be incorporated into spermicides, incorporated into or attached to surfaces of condoms, materials from which surgical gloves, dressings and other medical equipment are made or attached to the surfaces of containers or other materials (e.g., filters) for receiving, processing and/or storing blood. In each case, the CD4 fragments of the present invention will bind to HIV gp120 envelope protein (and, thus, to HIV), which will be prevented from further passage (e.g., in the case of spermicides, condoms) or can be removed (e.g., in the case of donated or stored blood).

It is reasonable to assume that the modified soluble CD4 fragments of the present invention with altered (i.e., enhanced gp120 binding ability) will be shown to have the same advantage described for soluble human CD4 fragments. That is, it is reasonable to assume such fragments of the present invention have the capacity to bind the HIV gp120 envelope protein and interfere with HIV infection of T cells, but will not interfere with the function or proliferation of human T lymphocytes which are not infected with HIV. The capability of fragments to bind gp120 envelope protein and interfere with HIV infection and their lack of interference with uninfected T lymphocytes can be assessed by means described herein.

Modified soluble human CD4 fragments having altered gp120 binding ability can be used for therapy, diagnosis and prevention of infection by HIV. For example, use of fragments having slightly reduced or turned down affinity may improve the

effective pharmokinetics of therapy. For example, such fragments can be used to bind or hold on to gp120 (and, thus, HIV) transiently. Such fragments bind the virus long enough to render it ineffective as an infectious agent and to prepare it to bind or accept another therapeutic agent (e.g., one which will destroy the virus).

In addition, the region of the CD4 domain I implicated as a possible binding site for gp120 is distinct from the loops analogous to hypervariable complementarity determining segments. If those loops form a binding site for class II MHC, the putative natural ligand of CD4, one can speculate that gp120 may be incapable of inhibiting class II recognition events, even after it has bound to the CD4 structure. Thus, the CD4 mutant described herein should be useful in future analysis of CD4 class II MHC interactions.

Fragments of the present invention having enhanced gp120 binding ability can be used therapeutically (in vivo) to treat individuals infected with HIV. Such fragments can be administered by an acceptable route (e.g., intravenously, intramuscularly, intraperitoneally, orally), alone or after combination with an acceptable carrier (e.g., saline buffer). Modified soluble CD4 fragments with enhanced gp120 binding ability of the present invention can be administered to inhibit binding of HIV to T4 lymphocytes and to inhibit HIV transmission from an infected cell to uninfected cells by interfering with syncytium formation. The quantity of such CD4 fragments administered will be determined on an individual

basis, but will generally range from approximately 10 ug/kg body weight to approximately 500 ug/kg body weight per day (in one or more doses per day).

Modified soluble CD4 fragments having enhanced gp120 binding ability can also be used for diagnostic purposes. Because of their enhanced binding ability, they can be used in known immunoassay procedures for detecting the presence and determining the quantity, if desired, of HIV gp120 envelope protein (and, as a result, of HIV itself) in samples, such as blood, semen and saliva. CD4 fragments of the present invention can be, for example, attached or bound by virtue of the CD4 fragment to solid support, such as latex beads, which are then contacted with a sample to be assayed, in such a manner that if HIV is present in the sample, it will be bound (by virtue of the CD4 fragment-gp120 interaction). This can be followed by precipitation and/or labelling through contact with an anti-gp120 antibody and detection of the precipitate or labelled product, using known techniques.

Modified soluble CD4 fragments having enhanced gp120 binding ability can also be used for the prevention of HIV infection. For example, such fragments can be incorporated in or attached to materials which might come in contact with HIV. They can be incorporated into spermicides; incorporated into or attached to surfaces of condoms, materials from which surgical gloves, dressings and other medical equipment are made; or attached to the surfaces of containers or other materials (e.g., filters) for receiving, processing and/or storing blood. In each case, the CD4 fragments of the

present invention will bind to HIV gp120 envelope protein (and, thus, to HIV), which will be prevented from further passage (e.g., in the case of spermicides, condoms, surgical gloves, dressings) or can be removed (e.g., in the case of donated or stored blood).

Example 1 Production of Soluble CD4 Fragments

Initially, cDNA encoding human CD4 was engineered in order to delete the nucleotide sequence encoding the hydrophobic transmembrane region, which ordinarily renders CD4 membrane bound and insoluble. As a result, cDNA encoding soluble human CD4 fragments was produced.

Plasmid Construction

Plasmid construction can best be described with reference to Figure 2. As shown in Figure 2, plasmid vector pAc373/$T4_{ex}$, containing the truncated CD4 gene, was constructed from plasmids pAc373 and pSP65-T4.

The plasmid transfer vector pAc373 contains a single BamHI cloning site 8 base pairs upstream of the polyhedrin ATG start site. In order to produce a secreted form of the CD4 molecule, the plasmid CD4 protein-encoding pSP65-T4 (kindly provided by Dan Littman, Univ. of California, San Francisco, CA) was digested with BamHI and XhoI to release the CD4 cDNA insert (which can be readily obtained as described in the literature, as in, for example, Madden et al. Cell, 42: 93-104 (1985)). The CD4 cDNA insert was subsequently digested with NciI, which cleaves CD4 cDNA at nucleotide positions 83, 1253 and 1604,

producing a fragment of 1.17Kb which lacks the ATG start codon and terminates just prior to the trans-membrane region.

Two oligonucleotides, 5'AGGATCCTTAATGAACC3' and 5'CGGTTCATTAAGGATCCT3', were synthesized, using standard cyanoethyl phosphoramidite chemistry. They were annealed and kinased to generate a linker molecule which reconstructs the ATG translation initiation codon, includes a stop codon (TAA) for termination of transcription, creates an NciI cohesive end, and adds a BamHI cloning site. Linkers were ligated to the 1.17Kb CD4-encoding fragment and then digested with BamHI to generate BamHI cohesive ends. Subsequently, the CD4-encoding fragment was inserted into the BamHI cloning site of the publicly available transfer vector pAc373. Recombinant plasmids containing a single copy of the truncated CD4 molecule in the correct orientation were identified by restriction mapping. The constructs were then sequenced by the ^{35}S-ATP labelled dideoxy method to confirm the expected sequence at the junctions of insertion. Recombinant plasmids pAc373/T4$_{ex1}$ and pAc373/T4$_{ex2}$ were characterized in detail. They contained identical 5' ends. The synthetic linker ligated in the expected orientation in pAc373/T4$_{ex1}$ to result in a predicted CD4 protein carboxy-terminus of LPTWSTPVH.

Transfer of the T4$_{ex}$ sequence from the plasmid vector to the Autographa californica nuclear polyhedrosis virus (AcNPV) genome was accomplished essentially as described in Smith et al. (1985) P.N.A.S. U.S.A. 82, 8404-8408. In this method, cotransfection by calcium phosphate precipitation of

4 ug pAc373/$T4_{ex}$ DNA with 1 ug of purified AcNPV DNA into S. frugiperda cells (SF9), which are publicly available, resulted in homologous recombination between the recombinant sequence of the transfer vector and the polyhedrin gene sequence of AcNPV. Recombinant AcNPV contains an inactivated polyhedrin gene which no longer forms occlusions in infected cells. For plaque purification, 2 x 10^5 SF9 cells were seeded in 100 mM Petri dishes approximately 24 hours prior to assay. Ten fold dilutions of viral supernatant were prepared using final media [Grace's insect medium (Gibco, Grand Island, NY), Difco TC yeastolate 0.33%, lactalbumin hydrosylate 0.33%, 2 mM supplemental glutamine and 50 ug/ml gentamycin containing 10% FCS (Hyclone, Loga, UT)]. Each plate was innoculated with 1 ml of virus (10^{-3} to 10^{-7} dilution) plus 2 ml of final media. After incubation for 2 hours, the innoculum was removed and replaced with 10 ml of 1.5% Sea Plaque agarose (FMC Bioproducts, Rockland, ME) in final media. After agarose solidification, plates were transferred to a humid environment for 4-6 days at 27°C.

Plaque assay of the transfection supernatant yielded plaques of distinct morphology: either infected cells which are occlusion positive (wild type AcNPV) or occlusion negative (recombinant CD4 virus). Occlusion-negative plaques were identified, selected, and further plaque purified. DNA from cells infected with putative CD4 recombinant virus was hybridized with a ^{32}P labelled CD4 cDNA probe to verify the presence of the CD4 sequence. Production of the $T4_{ex}$ polypeptide was carried out as follows: 6 x 10^5 SF9 cells were seeded per well in 24 well

Nunc plates (Interlab, Thousand Oaks, CA) for 2 hours at 27°C and then adherent cells infected with virus at an MOI of 10 in 0.2 ml final media for 2 h. The innoculum was then removed and cells cultured in 0.5 ml fresh medium at 27°C for 48 hours. Adherent cells were then washed twice with 0.5 ml Grace's medium lacking serum and methionine followed by incubation in 0.5 ml in the same medium for 1 hour. The adherent cells were washed once and then cultured for 6 hours in serum and methionine-free Grace's medium containing 67 uCi ^{35}S methionine (New England Nuclear, Boston, MA 1134 Ci/mmol). Culture supernatants were harvested, microfuged for 10 minutes, and dialyzed at 4°C against PBS containing 0.05% sodium azide and 10 mM cold methionine. Cells were dislodged from the wells, washed twice with Grace's medium at 4°C (by centrifugation in a Sorvall RT6000 for 5 minutes at 1000 rpm) and finally lysed for 30 minutes at 4°C by the addition of a RIPA buffer containing 1% Triton X-100, 0.15 M NaCl and a cocktail of protease inhibitors (see below).

The lysates were microfuged for 10 minutes and dialyzed at 4°C as for culture supernatants. Both lysates and culture supernatants were subjected to immunoprecipitation for 16 hours at 4°C with a monoclonal anti-CD4 antibody (19Thy5D7) linked to Affigel-10 beads (5 mg monoclonal antibody/ml gel). After immunoabsorption, the beads were washed twice with lysis buffer and bound material was eluted by treatment of the beads with 0.1 M glycine-HCl buffer, pH 2.0. Eluates and whole samples of lysates or culture supernatants were mixed with SDS sample buffer containing 2-mercaptoethanol, boiled for 5

minutes and electrophoresed in 12.5% mini-slab gels according to Laemmli. Subsequently, the gels were fixed, dried and autoradiographed using Kodak XAR-5 film. High titer viral stocks were generated by infecting SF9 cells at an MOI of 1 and culturing at 1×10^6 cells/ml for 4 days in final media. These stocks were used for infecting SF9 cells for production of protein. For large scale production of protein, SF9 cells were grown in 2 liter spinner flasks in final media. Cells were harvested and infected with an MOI of 15 (using high titer viral stocks) at a concentration of 10×10^6 cells/ml. Cells were then pelleted, resuspended in media at 1×10^6/ml, and cultured for 3 days at 27°C in spinner flasks. At this time, supernatants were collected by centrifuging cultures to remove cells.

For large scale purification, infected SF9 cell culture supernatants were harvested by centrifugation of cells in a Sorvall H-4000 rotor at 800 rpm for 6 minutes at 4°C. The culture supernatants were then subjected to protease inhibition by the addition of a cocktail of protease inhibitors made up of leupeptin, antipain, pepstatin, and chymostatin to final concentrations of 0.5 ug/ml; soybean trypsin inhibitor to 0.02 ug/ml; and phenyl methyl sulfonyl fluoride (PMSF) to 1.25 mM, followed by adjustment of the pH to 6.8 by the dropwise addition of 1 M NaOH. The samples were subsequently clarified by centrifugation in a Sorvall GSA rotor at 8000 rpm for 25 minutes at 4°C and pumped at 4°C at a flow-rate of 30 ml/hour through a 2 ml precleared immunoabsorbent column, 21Thy2D3 monoclonal antibody (anti-T8) coupled to Affigel-10 (Biorad), followed in series by a 7 ml

column of anti-CD4 monoclonal antibody (19Thy5D7) coupled to Affigel 10 at a concentration of 7.5 mg monoclonal antibody per ml of gel; the monoclonal antibodies were made according to conventional methods. The anti-CD4 column was then washed with 30 ml of 10 mM Tris-HCl buffer, pH 5.0. The bound CD4 polypeptides were eluted by pumping 0.1 M glycine-HCl, pH 2.0, through the washed anti-CD4 column and 0.8 ml fractions of eluant were collected into tubes containing 0.15 ml 1 M Tris-HCl, pH 7.6. During the whole column fractionation procedure, eluate absorption was monitored at 280 nm with a Uvicord 2 (LKB, Gaithersburg, MD) fitted with an event marker. Fractions of neutralized pH 2.0 eluate containing protein were pooled and concentrated by ultrafiltration in a stirred cell (Amicon, model 3) fitted with a YM-5 membrane. Typically, the yield of purified $T4_{ex}$ polypeptides was 1 ug/ml of infected SF9 culture supernatants. Aliquots containing 1 ug of protein concentrate (assuming that 1 OD unit = 1 mg/ml at a 280 nm) were examined for purity in 12.5% SDS-polyacrylamide slab gels, stained with Coomassie blue.

<u>Purification and characterization of the $T4_{ex}$ polypeptides</u>

SF9 cells infected with either recombinant baculovirus containing the $T4_{ex}$ cDNAs or wild type AcNPV virus were cultured in ^{35}S-methionine and products were examined by SDS-PAGE, followed by autoradiography.

It was shown that the $T4_{ex1}$ polypeptide is the major secreted product of SF9 cells infected with the

$T4_{ex1}$ recombinant baculovirus. The predominant ^{35}S labelled protein band (45% of total labelled material) in SDS-PAGE analysis of supernatants from SF9 cells obtained 54 hours after $T4_{ex1}$ recombinant baculoviral infection is a 50KD band under reducing conditions. This band co-migrates with material immunoprecipitated by anti-CD4 monoclonal antibody (19Thy5D7) from $T4_{ex1}$ baculovirus infected SF9 supernatants or cell lysates. In addition, the latter shows a strongly labelled band of 52KD which presumably represents the $T4_{ex1}$ polypeptide still carrying the uncleaved signal peptide. Although a 50KD band is readily detected in the total cell lysate of $T4_{ex1}$ virus infected cells even in the absence of immunoprecipitation with anti-CD4 monoclonal antibody, it is a minor component of a complex mixture of labelled intracellular polypeptides. As expected, no CD4 material was precipitated from supernatants of wild type AcNPV-infected cells or detectable in the total supernatant.

Each of two representative $T4_{ex2}$ preparations yielded a protein that migrated under reducing conditions with a molecular weight of 51KD (and is glycosylated as indicated by endoglycosidase F experiments), whereas the $T4_{ex1}$ protein migrated slightly faster with a molecular weight of 50KD. These different mobilities in SDS-PAGE between $T4_{ex1}$ and $T4_{ex2}$ proteins are not unexpected, since $T4_{ex2}$ contains 17 additional carboxy terminal amino acids derived from fusion with the polyhedrin gene. Under nonreducing conditions, the mobility of $T4_{ex1}$ protein is faster than under reducing conditions, consistent

with previous predictions that there are intrachain disulfide bonds in the CD4 external segment, and also showing the absence of covalent disulfide linked polymers of $T4_{ex1}$ protein. The protein production strategy described above routinely yields 1-2 mg of secreted $T4_{ex1}$ or $T4_{ex2}$ proteins per liter of SF9 cells (1-2 x 10^9 cells) over a 72 hour culture period.

To verify that the 50KD $T4_{ex1}$ and 51KD $T4_{ex2}$ proteins were indeed the products of the CD4 gene, purified polypeptides were electroblotted onto polyvinylidene difluoride membranes (Millipore, 0.45 mum pore size) and the Coomassie blue stained 50-51KD material subjected to amino terminal sequencing on an Applied Biosystems model 470A sequenator equipped with an on-line 120A PTH analyzer using the 03R PTH program. In each case, the first 10 cycles yielded the unambiguous sequence: KKVVLGKKGD. In contrast, the predicted N-terminal sequence of CD4 based on translation of the cDNA nucleotide sequence previously has been suggested to be either QGNKVVLGKKGD or NKVVLGKKGD. The second of these two assignments was based on homology with the rat N-terminal sequence KTVVLGK. While the empirically derived sequences herein are consistent with the positioning of latter N-terminal human assignment and that of CD4 in mouse and sheep, the K at position 1 assigned by the present amino acid sequence analysis is at variance with the amino acid predicted from the nucleotide sequence of the cDNA. To resolve these differences, DNA sequencing of pAc373/$T4_{ex2}$ inserts was carried out. The codon for the amino terminal residue was determined to be AAG, rather than AAC, as given in

the original CD4 cDNA cloning paper (Madden _et al._, _id_) without other differences noted. Whether this single nucleotide discrepancy represents a mutation resulting from cloning into pAc373 is not known, but appears unlikely in view of the lysine residue found at the N-terminus of the homologous rat CD4 sequence. From these data, it was concluded that the amino terminus of mature human CD4 begins with two lysine residues, followed by two valine residues and that $T4_{ex1}$ and $T4_{ex2}$ are CD4 derived polypeptides. In addition, this data shows that the baculovirus expression system has the capacity to enzymatically cleave the signal peptide from the $T4_{ex}$ polypeptide precursor, allowing it to be secreted. Thus, the hydrophobic transmembrane portion of the CD4 protein, which ordinarily causes the protein to be insoluble, is deleted, as are the first three or four external amino acids adjacent the transmembrane portion. This means that the truncated soluble CD4 polypeptides have 371 amino acid residues ($T4_{ex1}$) or 370 amino acid residues ($T4_{ex2}$), compared to the 374 amino acid mature extracellular segment.

Binding of Soluble CD4 Fragments to HIV gp120

To determine whether the soluble CD4 proteins produced in the baculovirus system could bind to the HIV gp120 exterior glycoprotein, the following two reciprocal coprecipitation experiments were carried out. First, metabolically labelled gp120 protein derived from HIV virions was incubated with un-labelled purified $T4_{ex2}$ either in the absence or presence of monoclonal antibodies directed against distinct epitopes of the CD4 protein (OKT4 and

OKT4A). The OKT4A antibody (like 19Thy5D7), but not the OKT4 monoclonal antibody, is known to inhibit the binding of gp120 to the CD4 molecule. Culture supernatants were collected from 1 x 10^7 Molt-3 lymphocytes stably infected with the HIV strain IIIB that were metabolically labelled overnight with 100 uCi/ml. ^{35}S-cysteine in total volume of 1.5 ml. NP-40 was added to a final concentration of 0.5% and the supernatants were incubated with 10 ug of $T4_{ex2}$ for 1 hour at 37°C, with or without preincubation of the soluble T4 with 5 ug/ml of OKT4A (Ortho Pharmaceutical, Raritan, NJ). The samples were then immunoprecipitated with the monoclonal antibody OKT4 and run on SDS polyacrylamide gels as described in Kowalski et al., Science, 237:1351-1355 (1987). In addition, 1.5 ml of unlabelled culture supernatants were collected from either 1 x 10^5 uninfected Molt-3 lymphocytes or HIV-infected Molt-3 lymphocytes and incubated for 1 hour at 37°C with ^{125}I labelled soluble T4 that had been radioiodinated by Bolton-Hunter reagent (NEN, Boston, MA). The samples were then immunoprecipitated using a goat anti-gp120 antiserum, as described in Kowalski et al., id.

In the absence of OKT4A monoclonal antibody preincubation, the gp120 protein was coprecipitated by OKT4 monoclonal antibody. In contrast, preincubation with OKT4A antibody inhibits gp120 co-precipitation. These findings show that gp120 binds to the $T4_{ex2}$ protein and that this binding is inhibited by OKT4A. The OKT4 monoclonal antibody did not precipitate the gp120 protein in the absence of added $T4_{ex2}$ protein.

In a second experiment, unlabelled HIV virions were incubated with radioiodinated $T4_{ex2}$ protein. The mixture was then immunoprecipitated with a goat antiserum raised against purified HIV gp120 protein. The iodinated $T4_{ex2}$ protein was coprecipitated by the anti-gp120 serum only when HIV virions were present, indicating that the $T4_{2x2}$ protein was capable of binding to an HIV virion component.

Inhibition of gp120 Binding to T4 Soluble CD4 Fragments

To examine whether the $T4_{ex1}$ protein could inhibit the binding of gp120 protein to CD4+ lymphocytes, metabolically labelled gp120 protein from the supernatants of virus-infected cells was preincubated with $T4_{ex1}$ protein or a control protein made in the baculovirus system (an extracellular T11 segment), as follows.

1×10^7 H9 lymphocytes stably infected with the HIV strain IIIB were metabolically labelled with ^{35}S-cysteine overnight in a total volume of 1.5 ml. The supernatants containing labelled HIV proteins were incubated for 1 hour at 37°C with either: phosphate buffer saline (PBS), 2.5 ug/ml OKT4A; 30 ug/ml $T4_{ex1}$; or 30 ug/ml T11. The SupT1 cells were centrifuged, washed once with PBS, lysed with 0.75 ml of lysis buffer and the gp120 bound to the SupT1 cells was immunoprecipitated as described in Kowalski et al., id. The OKT4 and OKT4A monoclonal antibodies were added to the SupT1 cells prior to the addition of the labelled protein to control for specificity of the binding.

The OKT4A but not the OKT4 monoclonal antibody was found to inhibit the binding of the labelled gp120 protein to the SupT1 cells. The $T4_{ex1}$ protein significantly inhibited the binding of labelled gp120 to the surface of SupT1 cells. No inhibition was observed using up to a 30 ug/ml concentration of the control protein, whereas inhibition of gp120 binding was seen at 0.5 ug/ml concentration of $T4_{ex1}$ protein.

Effect of Soluble CD4 Fragments on HIV Replication

To determine whether the $T4_{ex1}$ polypeptide would inhibit infection of cells by HIV, experiments were carried out using two types of lymphocytes (C8166 and H9). For each cell type, 2×10^5 cells were infected with 1000 $TCID_{50}$ units of the $H9III_B$ strain of HIV, in the presence of proteins, which were added at the time of infection and maintained at the following concentrations throughout the course of the experiment: no added protein; ovalbumin (control), 10 ug/ml; $T4_{ex2}$ protein, 10 ug/ml; $T4_{ex2}$ protein, 2 ug/ml; $T4_{ex2}$ protein, 0.2 ug/ml; 21 Thy2D3 (anti-CD8, control), 10 ug/ml; and 19Thy5D7 (anti-CD4, control), 0.2 ug/ml. On day 9 following infection, supernatants were collected and assayed for viral p24 gag protein by radioimmunoassay. Referring to Figure 4, the amount of p24 protein in cell supernatants is plotted vs. the concentration of added ovalbumin (□), soluble T11 produced in baculovirus (■), $T4_{ex1}$ protein (○), $T4_{ex2}$ protein (●) or 19Thy5D7 ( ).

As shown in Figure 4, while ovalbumin, recombinant T11 and 21 Thy2D3 proteins exerted no effect on virus replication, ug/ml concentrations of

$T4_{ex1}$ and $T4_{ex2}$ and anti-T4 (19Thy5D7) exhibited significant inhibition of viral protein expression and virus production. The $T4_{ex1}$ and $T4_{ex2}$ proteins were able to decrease HIV p24 protein expression at concentrations of 0.2 ug/ml. These studies indicate that the $T4_{ex1}$ and $T4_{ex2}$ proteins inhibit HIV replication in CD4+ lymphocytes.

Inhibition of HIV Envelope-induced Syncytia by Soluble CD4 Fragments

The induction of syncytia by the HIV envelope depends upon binding of the gp120 exterior glycoprotein to the CD4 molecule, followed by events involved in membrane fusion. To examine whether the $T4_{ex2}$ protein could inhibit the formation of syncytia by the HIV envelope, cells chronically infected with HIV were cocultivated with CD4+ SupT1 lymphocytes in the presence or absence of the $T4_{ex2}$ protein. Addition of control proteins, ovalbumin, or an anti-T8 monoclonal antibody (21Thy2D3) to the cocultivated cells had no effect on the formation of syncytia, which were scored at 6 h after the cocultivation had begun (Table 2). By contrast, addition of as little as 2 ug/ml of $T4_{ex2}$ or anti-T4 (19Thy5D7) was able to completely inhibit the formation of syncytia in this assay. Both $T4_{ex1}$ and $T4_{ex2}$ proteins inhibited the induction of syncytia when CHO cells constitutively expressing the HIV envelope were cocultivated with SupT1 lymphocytes. No inhibition of syncytium formation was observed with a recombinant secreted T11 protein made in a baculovirus expression system.

TABLE 2

INHIBITION OF HIV ENVELOPE-INDUCED SYNCYTIA BY SOLUBLE T4 PROTEINS

Protein	Concentration (μg/ml)	Syncytia
None	-	—
Ovalbumin	50	—
	20	—
	5	—
	2	—
Anti-T3 (21Thy2D3)	50	—
	20	—
	5	—
	2	—
Anti-T4 (19Thy5D7)	50	-
	20	-
	5	-
	2	-
T4ex2 protein	50	-
	20	-
	5	-
	2	-

Approximately 5×10^5 SupT1 CD4+ lymphocytes were cocultivated with 1×10^4 Jurkat lymphocytes infected with the HXBc2 strain of HIV in 1 ml volume in the presence of the indicated concentrations of protein. Ten hours after beginning of cocultivation, dilutions were made and number of syncytia per milliliter estimated (——, >1000; +—, 500-1000; +, 50-500; -, <50).

To assess whether the observed inhibition of syncytia by the $T4_{ex2}$ product was due to its interaction with the envelope-expressing cells or with the CD4+ "target" cells, either of these cells was separately incubated with the $T4_{ex2}$ protein, washed, and then used in the cocultivation assay. Table 3 shows that the pretreatment of the envelope-expressing cells (H9/HTLVIIIB) was as effective at syncytia inhibition as was pretreatment of both envelope-expressing and CD4+ (SupT1) cells. In contrast, incubation of the "target" SupT1 cells with the $T4_{ex2}$ protein exhibited only slight effects on syncytium formation. Thus, the soluble CD4 fragments appears to exert their syncytium-inhibiting effect through its interaction with the envelope-expressing cells.

TABLE 3

INHIBITION OF SYNCYTIUM FORMATION BY INTERACTION

WITH ENVELOPE-EXPRESSING CELLS

	Number of syncytia	
	SupT1 + ovalbumin	SupT1 + $T4_{ex}2$
H9/HTLVIIIB + ovalbumin	1560	1120
H9/HTLVIIIB + $T4_{ex}2$	130	120

Approximately 1 x 10^6 H9/HTLVIIIB lymphocytes or 4 x 10^6 SupT1 lymphocytes were incubated in medium with either ovalbumin or $T4_{ex}2$ protein at a concentration of 20 ug/ml at 37 C for 30 min. The cells were then centrifuged and washed with phosphate buffered saline, centrifuged and resuspended in medium. The treated H9/HTLVIIIB were then mixed with the treated SupT1 cells in 24 well dishes and returned to a 37°C, 5% CO_2 incubator for 5 h, when total syncytia per well were counted.

Lack of Inhibition of Class II MHC Recognition Events by Soluble CD4 Fragments

Because CD4 function is necessary for facilitating activation of class II specific CTL and Ia restricted helper T lymphocytes, experiments were carried out to examine whether the same $T4_{ex}$ proteins abrogate physiologic response of CD4+ T lymphocytes. In this regard, two types of experiments were performed. The first examined the effect of $T4_{ex2}$ on class II MHC directed killing mediated by the CD4+ cytolytic clone AA8. The T4+ cytolytic clone AA8 is specific for class II MHC gene products on the allogeneic EBV transformed B cell line Lax 509. Referring to Figure 3, ^{51}Cr labelled Lax 509 cells were preincubated with secreted $T4_{ex2}$ (△) or a control protein (BSA (□) for 30 min at 4°C prior to addition of effector cells. In other wells, anti-T4 (19Thy5D7) (○) and anti-CALLA J5 (kind gift of Jerome Ritz, Dana Farber Cancer Institute, Boston, MA) (●) antibodies were used as inhibitors. After preincubation of targets (3000 cells/well) with secreted CD4 fragment inhibitors, lysis was measured in a standard 4h ^{51}Cr release assay at an E/T ratio of 30:1. Results shown are the mean of quadruplicate samples where standard deviations are greater than 10%.

As shown, $T4_{ex2}$, like the control protein BSA and the anti-T8 monoclonal antibody, failed to inhibit CTL effector function even at concentrations as high as 100 ug/ml. In contrast, as little as 1-3 ug/ml of specific anti-T4 (19Thy5D7) monoclonal antibody reduced cytolysis by less than 50%. $T4_{ex1}$ also was without effect on cell lysis.

Lack of Effect on T-Cell Proliferation by Soluble CD4 Fragments

The following experiment was carried out to determine the effect of $T4_{ex1}$ on proliferation of the T4+ tetanus toxoid specific, class II MHC restricted helper T cell clone CTT7. Referring to Figure 6, for proliferative studies, 50,000 cells/well of the tetanus toxoid specific clone CTT7, derived by standard cloning strategies, was cultured in 10% FCS/RPMI 1640 supplemented with 1% pen-strep and 2% glutamine alone, with 10 ug/ml of tetanus toxoid (Massachusetts Department of Public Health, Jamaica Plain, MA) or the combination of tetanus toxoid (TT) and 10% autologous macrophages (MO) in the presence or absence of a final concentration of 40 ug/ml ovalbumin (ova), anti-T4, anti-T8, or twice immunoabsorbed $T4_{ex1}$ for 24 h at 37°C in a humid atmosphere with 6% CO_2. Subsequently, wells were pulsed with 1 uCi/well f ^{3}H-TdR. Cells were harvested at 48 h using an automated cell harvester. Plus signs (+) indicate presence of a given additive.

As shown in Figure 6, the CTT7 clone is activated to undergo proliferation only in the presence of tetanus toxoid and the autologous antigen presenting cell. At a concentration of 40 ug/ml, the anti-CD4 (19Thy5D7) monoclonal antibody inhibited ^{3}H-TdR incorporation by 80%, consistent with the important role of CD4 in helper T cell response. In contrast, equivalent amounts of $T4_{ex1}$, ovalbumin, or anti-T8 monoclonal antibody have no effects.

Thus, while $T4_{ex}$ protein binds HIV gp120 and thereby inhibits binding of gp120 to its receptor, HIV envelope-induced syncytium formation and HIV

replication, it has no discernable effect on class II directed physiologic T cell response at identical concentrations under these experimental conditions. The basis for this difference remains to be resolved. One possibility is that the affinity of CD4 for gp120 is substantially higher than CD4 for its native ligand (presumably class II MHC). In addition, because CD4 is only one of several elements (others including LFA-1, T11, etc.) that facilitate cell-cell interactions between CTL and targets or inducer T cells and antigen presenting cells, partial abrogation of the CD4 function with $T4_{ex1}$ protein may still leave the T cell activation process uninhibited.

Thus, concentrations of soluble CD4 fragments in the picomolar range, like certain anti-T4 monoclonal antibodies, inhibit syncytium formation and HIV infection. However, in contrast to anti-T4, the effects of soluble CD4 protein are exerted at the level of gp120 expressing cells. In addition, class II specific T cell interactions are functionally unimpeded by soluble CD4 protein, whereas they are virtually abrogated by equivalent amounts of anti-T4 antibody under the same experimental conditions. Whether this selective effect is a consequence of substantial differences in CD4 affinity for gp120 compared to antibody remains to be determined. Nevertheless, the present findings indicate that the extracellular segment of the CD4 protein or peptide fragments derived from it can be useful in competitively inhibiting the interaction between the native transmembrane CD4 structure on T lymphocytes and the viral gp120 protein. Furthermore, these soluble CD4 proteins should allow the establishment of assays

designed to detect drugs which might interfere with gp120-CD4 interactions. Importantly, $T4_{ex}$ proteins themselves or fragments derived from $T4_{ex}$ may have clinical utility in inhibiting gp120 binding to membrane bound CD4 on T lymphocytes, monocytes, or brain cells without interfering with the normal physiological role of surface CD4 on healthy cells.

Example 2 Assessment of Interaction of $T4_{ex}$ Proteins and Derived Peptide Fragments with HIV gp120

To further analyze the specific physical interaction between $T4_{ex}$ proteins or their derived peptide fragments and gp120, a method was employed which involved size fractionation by SDS-PAGE followed by electroblotting of the $T4_{ex2}$ protein onto polyvinyladine diflouride membranes. $T4_{ex2}$ (75 ug in neutralized immunoaffinity eluate) was mixed with a 1/9 volume of 0.9 volume of 0.9 M Tris-HCL, pH 8.0 containing 40 mM $CaCl_2$. TPCK trypsin (Worthington) was added to an enzyme:protein ratio of 1:50 (w/w) and digestion was carried out at 37°C. 25 ug aliquots were removed at 10, 20 and 45 minutes. Digestion was stopped by the addition of a non-reduced SDS sample buffer and heating to 100°C for 5 minutes. Aliquots were electrophoresed on 12.5% mini slab gel under non-reduced conditions. Gels were subsequently electroblotted, using the method of Matsudaira, onto polyvinylidene diflouride memebrane (Millipore; 0.45 um pore size). Mastsudaira, P., J. Biol. Chem., 262: 10035 (1987). Duplicate tracks were either stained with Coomassie blue or blocked with a 5% dried milk solution in PBS/azide for 2

hours at room temp. Electroblots after blocking were assembled in a miniblotter apparatus (Immunetics) and slots overlying the appropriate tracks filled with 50 ul of purified, native HIV gp120 at 20ug/ml. in 1% dried milk in PBS for testing for HIV gp120 reactivity. Incubation with shaking was carried out overnight at 4°C. Following three 5 minute washes with PBS/0.05% Triton X-100, the blots were incubated with radioiodinated mouse monoclonal IgG, anti-HTLVIII gp120 2.6 ug (specific activity, 2uCi/ug) diluted in 25 ml 1% milk in PBS for 1 hour at room temperature, then 1 hour at 4°C.

After five further washes with PBS/Triton X-100 and two washes of PBS the blots were air dried and autoradiographed at -70°C (using preflashed Kodack XAR film and an enhancer screen). Where appropriate, stained bands of interest were cut out and sequenced on a gas phase protein sequencer (Applied Biosystem 470A) with on line PTH analyzer (120A) using the 03RPTH programs.

The single band of $T4_{ex2}$ at 50KD MW when electrophoresed unreduced was shown to be capable of binding HIV gp120 strongly. In contrast, identical amounts of $T4_{ex2}$, either reduced or reduced and amidomethylated, did not bind gp120 when similarly examined. The lack of gp120 binding to reduced and alkylated $T4_{ex2}$ is not due to the modification during alkylation of the cysteine residues themselves, as shown by the concurrent lack of gp120 binding to reduced $T4_{ex2}$. The fact that the electrophoretic mobility of the $T4_{ex2}$ protein after reduction is slower than when not reduced is consistent with the prediction that there are intrachain disulfide bonds

in the external segment of human T4. In addition, the migration of the non-reduced $T4_{ex2}$ protein as a single moiety of 50KD shows that the purified protein does not contain disulfide-linked polymers of $T4_{ex2}$ protein. Identical results to the above were obtained when using $T4_{ex1}$ proteins. Taken together, these results demonstrated that under these conditions, it is likely that the binding of gp120 to $T4_{ex}$ proteins is dependent on the presence of intact disulfide bridges within the $T4_{ex}$ protein, which are presumably stabilizing the tertiary structure of their binding region.

Enzymic fragmentations on the purified $T4_{ex}$ protein carried out as described above produced a wide range of fragments. HIV gp120 binding analysis of blotted material from a 45 minute papain digest demonstrates that, in addition to the expected binding by the 50KD residual $T4_{ex1}$ protein, a fragment is present with a mobility of 28KD which binds gp120. In order to definitively identify and purify the 28KD fragment, 40-fold more $T4_{ex1}$ protein was digested with papain and separated by preparative SDS-PAGE. A portion of the blot was subjected to analysis for gp120 binding, and comparison of densitometric scans of the stained blot and the autoradiograph showed that the 28 kD material boudn a relative amount of gp120 similar to that bound by the residual $T4_{ex1}$ protein in the same track. Thus, the 28KD fragment bound HIV gp 120 with the same efficiency as the parent $T4_{ex2}$ protein. The 28 KD band was excised from the Coomassie blue stained portion of this same blot and subjected to amino terminal microsequencing. The first 11 cycles

yielded a single unambiguous sequence of KKVVLGKKGDT, showing that the 28KD fragment is an intact polypeptide chain derived from the amino terminal region of the $T4_{ex1}$ protein. Assuming an average MW for each amino acid of 110 daltons, the papain cleavage of $T4_{ex1}$ yielding the 28KD fragment can be located as being C-terminal to the cysteine residue at position 159 and with domain 3 proximal to the oligosaccharide addition sites at positions 256 and 300 (Figure 7C). Thus, the binding of HIV gp120 to $T4_{ex1}$ does not involve the C-terminal stretch of amino acids in domain 3 containing both N-linked glycosylation sites of the T4 structure, or domain 4. This result defines the gp120 binding portion of T4 as being in the N-terminal region of the protein.

Similar experiments utilized trypsin fragmentation of $T4_{ex}$ to further define the nature of the gp120 binding fragments. Digestion of $T4_{ex2}$ protein with trypsin produces a set of fragments different from those seen with papain digestion. Analysis of separated material from the 45 minute tryptic digest for gp120 binding shows only a weak signal produced by the trace amount of 50KD $T4_{ex2}$ protein left after digestion. Amino terminal sequencing of the strongly Coomassie staining 45 KD heterogeneous band derived from blots of the 45 minute digest shows the presence of two major sequences corresponding to tryptic cleavage at lysine residues 7 and 75 and a minor sequence corresponding to a cleavage at lysine 72 (Fig. 7c). No other signals are seen in this material, indicating that these three lysine residues are highly labile towards trypsin and that such cleavage and/or loss or

residues 1-7 is sufficient to abrogage the binding of pg120.

In order to investigate the possibility that a gp120 binding tryptic $T4_{ex2}$ fragment could be obtained using a more restricted digestion, the material migrating in the 40-45 KD region from a 20 minute tryptic digest of $T4_{ex2}$ was examined, and no binding ff gp120 was apparent. Microsequencing of the 45KD material derived from the 10 minute tryptic digest gave a mixed sequence, with two major signals present, one corresponding to cleavage at lysine 72. In addition, there were two minor signals, one derived from cleavage at lysine 7 and the other to cleavage at lysine 75. This information leads to the conclusion that probably the most labile tryptic residue in $T4_{ex}$ is the lysine at 72, and that residues 1-7 are not alone responsible for HIV gp120 binding to CD4.

That the only detectable alterations of $T4_{ex}$ protein after trypsin cleavage are in domain 1, and these pertubations are capable of inhibiting pg120 binding, argues strongly for an essential role of the native domain 1 region. The ability of the NH_2 -terminal 28KD papain fragment of $T4_{ex1}$ to bind HIV gp120 is consistent with this view. Given the requirement of an intrachain disulfide bond to maintain the native conformation of Ig-like domains, the loss of HIV gp120 binding after $T4_{ex1}$ or $T4_{ex2}$ reduction further supports the notion.

Further binding site information is provided by the observation that the same restricted tryptic cleavage is sufficient to destabilize the antigenic epitope recognizxed by anti-T4 monoclonal antibody

19Thy5D7. Thus, when tryptic digests of both $T4_{ex}$ proteins are passed through a 19Thy5D7 immunoabsorbant, only the residual intact $T4_{ex}$ protein is bound. The fact that 19Thy5d7 is known to inhibit HIV gp120 binding to CD4 makes this result not unexpected and suggests that the HIV gp120 binding region and the 19Thy5D7 epitope localize to the immunoglobulin V-like NH_2 -terminal segment of CD4. However, given the large size of the antibody molecule relative to T4 domain 1 and, thus, the potential for steric blockage, the location of the 19Thy5D7 epitope relative to the gp120 site cannot be accurately known.

Conservation of cysteines and multiple other invariant Ig residues in the T4 sequence, as well as secondary structure predictions, argue that the first 92 residues of the T4 molecule have similar tertiary structure to an Ig V domain. It thus could be expected that the lysine residues at positions 7, 72 and 75 are clustered in space near each other, extending from the surface of the domain. In the case of lysine residues 72 and 75, which fall within a highly conserved area of sequence bounded by a asparate residue (Figure 7C, amino acid 78) and arginine residue (Figure 7C, amino acid 54) that probably form a salt bridge characteristic of V domains, this alignment is almost certainly correct. This cluster may be involved in the binding of HIV gp120 to the CD4 modecule.

The above results indicate that a linear stretch of amino acids is unlikely to be an effective, high affinity inhibitor of CD4-HIV gp120 interaction; the results show that disulfide bridging of $T4_{ex}$ protein

plays a key role in such interaction. The finding that cleavage at lysine residue 72 (i.e., between the cysteine residues in the V-like domain) is sufficient to destabilize both the gp120 and 19Thy5D7 binding region is consistent with this view. In addition, although these results implicate the V-like domain in the binding of gp120 to T4, the possibility has not as yet been ruled out that domain 2 containing cysteine residues at poisitions 130 and 159 (Figure 7C) might play a part in conjunction with the V-like domain 1.

Example 3 Production of Biologically Active, Modified Soluble Human CD4 Fragments and Assessment of Their Activity

Modifications of the T4 cDNA were produced using an M13 T4 template. The $T4_{ex}$ cDNA fragment was excised from plasmid vector pAc373/$T4_{ex}$ using BamHI. The plasmid vector pAc373/$T4_{ex}$ is described in Example 1 and in Hussey, et al., Nature, 331:78 (1988). The ends of the fragment were blunted with DNA polymerase I and the fragment ligated to XbaI linkers (New England Biolabs). The linkered fragment was digested with XbaI, gel purified to remove excess linkers and ligated to Xba cut M13 replicative form. The ligation mixture was transformed into competent TG1 host bacteria, plated out and the resulting plaques were screened by hybridization to T4 oligonucleotides. Plaques hybridizing to sense oligonucleotides were grown up to produce single-stranded M13 templates for mutagenesis.

The mutagenesis protocol is that marketed by Amersham and is based on the method of Eckstein

(Taylor, et al., Nucleic Acids Research, 13:8749 (1985); Taylor, et al., Nucleic Acids Research, 13:8764 (1985); Nakayama and Eckstein, Nucleic Acids Research, 14:9679 (1986)). Oligonucleotides were produced containing in their sequence a base change which, when incorporated, produced a stop codon, resulting in a truncated T4 protein. This resulted in truncation of the T4 molecules at amino acid #183. An oligonucleotide comprising the sequence 5' G-AAG-GCC-TAA-AGC-ATA-G was synthesized. The normal T4 sequence is G-AAG-GCC-TCC-AGC-ATA-G. Thus, the serine encoded by TCC was mutated to a stop codon TAA and the mutant T4 protein terminated at this point.

The mutant oligonucleotide was kinased and hybridized to 10 ug of the M13 T4 template. A second strand of DNA was synthesized, using the M13 T4 template and oligonucleotide primer, by the Klenow fragment of DNA polymerase in the presence of the thionucleotide dCTPαS. Any unreplicated single stranded DNA was removed by filtration through a nitro-cellulose filter and the purified double-stranded DNA was nicked with the restriction enzyme NciI. NciI will not cut phosphorothioate DNA. Thus, the new strand containing dCTPαS and the mutation were not nicked. The nicked DNA was digested with exonuclease III, which digested away the nicked, non-mutant DNA strand.

The gapped DNA was repolymerized by DNA polymeraseI, in the presence of T4 DNA ligase. In this step, the mutant strand served as the template so the mutation was copied into both strands.

The resulting DNA was transformed into competent TG1 and derived plaques were grown up. Single

stranded and replicative form DNAs were isolated and the DNA was sequenced to confirm the presence of the mutation. Mutated DNA was excised from the replicative form of DNA with Xba and ligated to Xba cut vector CDM8. This vector was developed and provided by Dr. Brian Seed (Massachusetts General Hospital). CDM8 is expressed in Cos cells upon transfection. Thus, the mutant T4 proteins could be assayed after transfection into Cos cells. CDM8 containing mutant T4 cDNAs were identified by hybridization to radiolabelled T4 DNA after transformation of the vector-insert ligation into competent MC1061P3 host bacteria. The proper orientation of insert in vector was determined by restriction enzyme analysis of mini-prep DNAs. Large scale plasmid preparations were used for transfection.

For transfections, 2-3 x 10^6 Cos cells were plated in 100 cm dishes in RPMI-10% FCS-1% glutamine-1% pen-strep-10 ug/ml gentamycin. 12-24 hours later, the cells were washed with RPMI and incubated for 2-2.5 hours in the presence of 4 ml DME containing 400 ug/ml DEAE-dextran and 45 ug plasmid DNA. The cells were washed with RPMI and incubated in 10 ml DME-2% FCS-1% glutamine-15 pen-strep-10 ug/ml gentamycin-120 uM chloroquine for 3 hours. The cells are washed with RPMI and incubated for 2 days in the original media.

EXAMPLE 4 Production of Modified Soluble Human CD4 Fragments

Methods: The 1.17Kb $T4_{ex1}$ fragment was excised using BamHI from pAC $373/T4_{ex1}$, blunted using the

Klenow fragment of DNA polymerase I, ligated to XhoI linkers (New England Biolabs) and subcloned in the XhoI site of the vector CDM8. Hussey, R.E. et al., Nature, 330:487-489 (1987); Seed, et al., Proc. Natl. Acad. Sci. U.S.A., 84:3365-3369 (1987) For transfection of CDM8 constructs into Cos cells, 2-3 X 10^6 cells are plated in 100 X 15 cm dishes in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS). Twelve to twenty-four hours later, 45 ug of plasmid DNA are added to 2.5 ml RPMI and mixed with 2.5 ml RPMI-800 ug/ml DEAE dextran, then added to the washed Cos cells. After approximately 2 hours at 37° C, the cells are washed and then incubated in RPMI containing 2% FCS, 1% glutamine, 1% penicillin-streptomycin, 10 ug/ml gentamycin and 150 uM chloroquine for 3 hours. The cells were incubated at 37° C for 2 days in RPMI 10% FCS. For metabolic labelling, the transfected Cos cells (2 days after transfection) and incubated for 1 hour in 5 ml RPMI minus cysteine containing 10% FCS. The media is removed and the cells are incubated in RPMI minus cysteine containing 10% dialyzed FCS and 100 uCi/ml of ^{35}S-cysteine for 5-6 hours at 37° C. The supernatants are removed, centrifuged at 200 g for 10 minutes and dialyzed vs. PBS/0.025% azide/10mM cold cysteine overnight at 4°C. For immunoprecipitation, 5 ml of the dialyzed ^{35}S-cysteine labelled supernatant is precleared by a 45 minute incubation at 4° C with 20 ul anti-T8 antibody (21Thy2D3) on Affigel-10 (Biorad) beads (about 5 mg antibody per ml beads). The precleared supernatant is then incubated with 20 ul anti-CD4 (19Thy5D7) on Affigel-10 beads for 3 hours at 4° C. The beads are washed once in 10

ml 10 mM Tris, pH 6.8/0.1% Triton X-100/0.1% SDS/0.5% DOC, once in ±1 ml of the same buffer and once in 1ml 0.1 M glycine, pH 5/0.1% Triton X-100 and then eluted with 35 ul 0.1 M glycine, pH 2/0.1% Triton X0100 and neutralized with 6 ul 1 M Tris, pH 7.6. The sample is run on a 0.75 or 1.5 mm 12.5% mini-polyacrylamide-SDS gel under non-reducing conditions. The gel is fixed, dried and autoradiographed at about 70° C from 1-7 days. Immunoprecipitation with anti-CD8 was carried out as above except that 20 ul anti-CD8 on Affigel-10 beads is used for immunoprecipitation. For co-precipitation with gp120 (kind gift of Dr. Bolognesi, Duke University), 0.5 ml of labelled supernatant is incubated with 67 ng native gp120 for 2 hours at 37° C. Five hundred ng anti-gp120 (Dupont) and 10 ul rabbit anti-mouse IgG Sepharose 4B beads are added and rotated for 2 hours at 4° C. The beads are washed once in 10 ml and once in 1 ml cold PBS, eluted and the sample run in SDS-PAGE as above.

The CD4 protein (182 amino acids long) was created using the thionucleotide method of oligonucleotide site directed mutagenesis. Taylor, J.W. et al., Nucl. Acids Res., 13:8749-8765 (1985); Taylor, J.W. et al., Nucl. Acids Res. 13:8765-8785 (1985); Nakayame et al., Nucl. Acids Res. 14:9679-9698 (1986) The XhoI insert of $T4_{ex1}$ was excised from CDM8, blunted with the Klenow fragment of DNA polymerase I ligated to XbaI linkers (New England Biolabs) and subcloned into M13mp18. Single stranded DNA was prepared as a template and mutagenesis was carried out according to the manufacturer's recommendations (Amersham). For the

182 amino acid truncation, the oligonucleotide 5' GAAGGCCTAAAGCATAG 3' was synthesized using standard cyanoethyl phosphoramodite chemistry. The termination codon which converts the serine (TCC) at amino acid 183 to a stop codon is underlined. The presence of the mutation was confirmed by sequencing the M13mp18-T4 construct and mini preps of the replicative form of the mutation-containing DNA were prepared. The mutated insert was excised with XbaI and ligated into the XbaI site of CDM8. The presence of the mutation was then directly confirmed by sequencing the CDM8-T4 insert using the double stranded DNA as a template. Although not shown, a truncation was also created at amino acid 110 using the oligonucleotide CACCTGCTTTAGGGGCAG.

EXAMPLE 5 Production and Analysis of CD4 Site-Directed Mutants

16 CD4 mutants were constructed, as described in Example 4. As shown in Table 1, 15 oligonucleotides were used, in a standard site-directed mutagenesis protocol (Example 4), to produce 16 different version of the human CD4 molecule, each containing from 1 to 4 amino acid substitutions. As a result, the amino acid residue normally present in human CD4 protein at the position indicated in Table 1 (See Figure 1) was replaced by the amino acid present in the equivalent position of the murine CD4 sequences.

Three mutants, M3, M9 and M14, evidenced altered gp120 binding ability: M3 failed to bind gp120, M9 has substantially reduced gp120 binding capacity and M14 also demonstrates reduced gp120 binding capacity.

The amino acid substitutions made in each are as follows:

M3 amino acid 48:P changed to G
amino acid 50:K changed to P
amino acid 51:L changed to S

M9 amino acid 121:P changed to S
amino acid 122:P changed to K
amino acid 123:G changed to V

M14 amino acid 155:G changed to D
amino acid 156:T changed to F
amino acid 158:T changed to N

P:	proline	S:	serine
K:	lysine	V:	valine
L:	leucine	D:	aspartic acid
G:	glycine	F:	phenylalanine
T:	threonine	N:	asparagine

<u>Equivalents</u>

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to be specific embodiments of the invention described herein. Such equivalents are intended to be encompassed within the scope of this invention.

CLAIMS

1. A soluble human CD4 fragment capable of binding to the gp120 envelope glycoprotein of human immunodeficiency virus.

2. A soluble human CD4 fragment of Claim 1 which does not interfere with the function and the proliferation of human T lymphocytes not infected with HIV, said soluble fragment including none of the transmembrane portion of CD4 or only a portion of said transmembrane region, said portion being sufficiently small that it does not prevent solubilization of said fragment.

3. A soluble human CD4 fragment which is capable of binding to the HIV gp120 envelope glycoprotein and which does not interfere with the function and the proliferation of human T lymphocytes not infected with HIV, comprising all or a portion of the amino acid sequence of Figure 1.

4. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected with HIV, the fragment differing from soluble human CD4 protein by a deletion from, substitution in or addition to the amino acid sequence of human CD4 protein of at least one amino acid.

5. A modified soluble human CD4 fragment, which is capable of binding HIV gp120 and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of the first 182 amino acids of human CD4 protein, as represented in Figure 1.

6. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of the first 369 amino acids of human CD4 protein.

7. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of domain 1, 2 and partial domain 3 CD4 protein.

8. A modified soluble human CD4 fragment of Claim 7 in which the amino acid sequence of soluble CD4 protein is truncated at amino acid position 243.

9. A modified soluble human CD4 fragment which is capable of binding HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, consisting essentially of soluble modified human CD4 protein in which: 1)

the asparagine present at amino acid position 271 in human CD4 protein is an aspartate and the asparagine present at amino acid 300 in human CD4 protein is an aspartate; 2) the asparagine present at amino acid position 271 in human CD4 protein is an aspartate; and 3) the asparagine present at amino acid 300 in human CD4 protein is an aspartate.

10. A biologically active, modified soluble human CD4 fragment encoded by a nucleotide sequence selected from the group consisting of:

a. the first 549 nucleotides of Figure 1;

b. the first 729 nucleotides of Figure 1; and

c. the first 1107 nucleotides of Figure 1.

11. A modified soluble human CD4 fragment, which binds HIV gp120 envelope protein and which does not interfere with the function and proliferation of human T lymphocytes not infected by HIV, which does not include the N-linked glycosylation sites present in the human CD4 fragment whose amino acid sequence is represented in Figure 1.

12. A soluble peptide, capable of binding HIV gp120, consisting essentially of domain I and domain II of human CD4 protein.

13. A soluble peptide, capable of binding HIV gp120, consisting essentially of 179 amino acids, said peptide having the amino acid sequence of domain I and domain II of human CD4 protein as represented in Figure 1.

14. Modified soluble human CD4 fragments having altered gp120 binding ability.

15. Modified soluble human CD4 fragments of Claim 14 having reduced gp120 binding ability.

16. Modified soluble human CD4 fragments of Claim 14 having enhanced gp120 binding ability.

17. DNA encoding a soluble human CD4 fragment which has the ability to bind HIV gp120 envelope protein.

18. DNA of Claim 17 encoding a soluble human CD4 fragment which additionally does not interfere with the function or proliferation of human T lymphocytes which are not infected by HIV.

19. DNA encoding a modified soluble human CD4 fragment which has the ability to bind HIV gp120 envelope protein.

20. DNA of Claim 19 encoding a modified soluble human CD4 fragment which additionally does not interfere with the function or proliferation of human T lymphocytes which are not infected by HIV.

21. DNA encoding a biologically active, modified soluble human CD4 fragment which does not include the N-linked glycosylation sites present in the human CD4 fragment whose amino acid sequence is represented in Figure 1.

22. DNA encoding a modified soluble human CD4 fragment capable of binding HIV gp120 envelope protein, said fragment consisting essentially of the first 182 amino acid of human CD4 protein.

23. DNA encoding a modified soluble human CD4 fragment capable of binding HIV gp120 envelope protein, said fragment consisting essentially of the first 369 amino acids of human CD4 protein.

24. DNA encoding a modified soluble human CD4 fragment in which the amino acid sequence is truncated at amino acid position 243, resulting in a CD4 fragment consisting essentially of domain 1, 2 and partial domain 3 CD4 protein.

25. DNA selected from the group consisting of: 1) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid position 271 in human CD4 protein is an aspartate and the asparagine present at amino acid 300 in human CD4 protein is an aspartate; 2) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid position 271 in human CD4 protein is an

aspartate; and 3) DNA encoding soluble modified human CD4 protein in which the asparagine present at amino acid 300 in human CD4 protein is an aspartate.

26. DNA comprising nucleotide triplets encoding human modified soluble CD4 protein, as represented in Figure 1, except that a nucleotide triplet encoding the amino acid present in the equivalent position of murine CD4 protein has been substituted at at least one nucleotide triplet site in the DNA, the triplet selected from the group consisting of:

a. the nucleotide triplet encoding the amino acid at position 48 of human CD4 protein;

b. the nucleotide triplet encoding the amino acid at position 50 of human CD4 protein;

c. the nucleotide triplet encoding the amino acid at position 51 of human CD4 protein; d.the nucleotide triplet encoding the amino acid at position 121 of human CD4 protein;

e. the nucleotide triplet encoding the amino acid at position 122 of human CD4 protein;

f. the nucleotide triplet encoding the amino acid at position 123 of human CD4 protein;

g. the nucleotide triplet encoding the amino acid at position 155 of human CD4 protein;

h. the nucleotide triplet encoding the amino acid at position 156 of human CD4 protein; and

i. the nucleotide triplet encoding the amino acid at position 158 of human CD4 protein.

27. A method of binding the HIV gp120 envelope protein _in vitro_, comprising contacting a fluid containing the HIV with a modified soluble CD4 fragment capable of binding the gp120 envelope protein, under conditions appropriate for binding of the gp120 envelope protein and the fragment to occur.

28. A method of binding HIV present in a biological sample, comprising combining the biological sample with a modified soluble CD4 fragment capable of binding the gp120 envelope protein of HIV, under conditions appropriate for binding of the fragment and the gp120 envelope protein to occur.

29. A condom comprising the modified soluble human CD4 fragment of Claim 4.

30. A composition comprising a spermicide and the modified soluble human CD4 fragment of Claim 4.

31. A device for blood collecting, blood processing and/or blood storage, comprising the modified soluble human CD4 fragment of Claim 4.

32. A medical garment, comprising the modified soluble human CD4 fragment of Claim 4.

FIGURE 1

```
AAGAAAGTGGTGCTGGGCAAAAAAGGGGATACAGTGGAACTGACCTGTACAGCTTCCCAG
----.----+----.----+----.----+----.----+----.----+----.----+  60 bp
TTCTTTCACCACGACCCGTTTTTTCCCCTATGTCACCTTGACTGGACATGTCGAAGGGTC

k  k  v  v  l  g  k  k  g  d  t  v  e  l  t  c  t  a  s  q
----.----+----.----+----.----+----.----+----.----+----.----+  20 aa

AAGAAGAGCATACAATTCCACTGGAAAAACTCCAACCAGATAAAGATTCTGGGAAATCAG
----.----+----.----+----.----+----.----+----.----+----.----+  120
TTCTTCTCGTATGTTAAGGTGACCTTTTTGAGGTTGGTCTATTTCTAAGACCCTTTAGTC

k  k  s  i  q  f  h  w  k  n  s  n  q  i  k  i  l  g  n  q
----.----+----.----+----.----+----.----+----.----+----.----+  40

GGCTCCTTCTTAACTAAAGGTCCATCCAAGCTGAATGATCGCGCTGACTCAAGAAGAAGC
----.----+----.----+----.----+----.----+----.----+----.----+  180
CCGAGGAAGAATTGATTTCCAGGTAGGTTCGACTTACTAGCGCGACTGAGTTCTTCTTCG

g  s  f  l  t  k  g  p  s  k  l  n  d  r  a  d  s  r  r  s
----.----+----.----+----.----+----.----+----.----+----.----+  60

CTTTGGGACCAAGGAAACTTCCCCCTGATCATCAAGAATCTTAAGATAGAAGACTCAGAT
----.----+----.----+----.----+----.----+----.----+----.----+  240
GAAACCCTGGTTCCTTTGAAGGGGGACTAGTAGTTCTTAGAATTCTATCTTCTGAGTCTA

l  w  d  q  g  n  f  p  l  i  i  k  n  l  k  i  e  d  s  d
----.----+----.----+----.----+----.----+----.----+----.----+  80

ACTTACATCTGTGAAGTGGAGGACCAGAAGGAGGAGGTGCAATTGCTAGTGTTCGGATTG
----.----+----.----+----.----+----.----+----.----+----.----+  300
TGAATGTAGACACTTCACCTCCTGGTCTTCCTCCTCCACGTTAACGATCACAAGCCTAAC

t  y  i  c  e  v  e  d  q  k  e  e  v  q  l  l  v  f  g  l
----.----+----.----+----.----+----.----+----.----+----.----+  100

ACTGCCAACTCTGACACCCACCTGCTTCAGGGGCAGAGCCTGACCCTGACCTTGGAGAGC
----.----+----.----+----.----+----.----+----.----+----.----+  360
TGACGGTTGAGACTGTGGGTGGACGAAGTCCCCGTCTCGGACTGGGACTGGAACCTCTCG

t  a  n  s  d  t  h  l  l  q  g  q  s  l  t  l  t  l  e  s
----.----+----.----+----.----+----.----+----.----+----.----+  120
```

FIGURE 1 (CONT'D)

```
CCCCCTGGTAGTAGCCCCTCAGTGCAATGTAGGAGTCCAAGGGGTAAAAACATACAGGGG  420
GGGGGACCATCATCGGGGAGTCACGTTACATCCTCAGGTTCCCCATTTTTGTATGTCCCC

p  p  g  s  s  p  s  v  q  c  r  s  p  r  g  k  n  i  q  g      140

GGGAAGACCCTCTCCGTGTCTCAGCTGGAGCTCCAGGATAGTGGCACCTGGACATGCACT  480
CCCTTCTGGGAGAGGCACAGAGTCGACCTCGAGGTCCTATCACCGTGGACCTGTACGTGA

g  k  t  l  s  v  s  q  l  e  l  q  d  s  g  t  w  t  c  t      160

GTCTTGCAGAACCAGAAGAAGGTGGAGTTCAAAATAGACATCGTGGTGCTAGCTTTCCAG  540
CAGAACGTCTTGGTCTTCTTCCACCTCAAGTTTTATCTGTAGCACCACGATCGAAAGGTC

v  l  q  n  q  k  k  v  e  f  k  i  d  i  v  v  l  a  f  q      180

AAGGCCTCCAGCATAGTCTATAAGAAAGAGGGGGAACAGGTGGAGTTCTCCTTCCCACTC  600
TTCCGGAGGTCGTATCAGATATTCTTTCTCCCCCTTGTCCACCTCAAGAGGAAGGGTGAG

k  a  s  s  i  v  y  k  k  e  g  e  q  v  e  f  s  f  p  l      200

GCCTTTACAGTTGAAAAGCTGACGGGCAGTGGCGAGCTGTGGTGGCAGGCGGAGAGGGCT  660
CGGAAATGTCAACTTTTCGACTGCCCGTCACCGCTCGACACCACCGTCCGCCTCTCCCGA

a  f  t  v  e  k  l  t  g  s  g  e  l  w  w  q  a  e  r  a      220

TCCTCCTCCAAGTCTTGGATCACCTTTGACCTGAAGAACAAGGAAGTGTCTGTAAAACGG  720
AGGAGGAGGTTCAGAACCTAGTGGAAACTGGACTTCTTGTTCCTTCACAGACATTTTGCC

s  s  s  k  s  w  i  t  f  d  l  k  n  k  e  v  s  v  k  r      240

GTTACCCAGGACCCTAAGCTCCAGATGGGCAAGAAGCTCCCGCTCCACCTCACCCTGCCC  780
CAATGGGTCCTGGGATTCGAGGTCTACCCGTTCTTCGAGGGCGAGGTGGAGTGGGACGGG

v  t  q  d  p  k  l  q  m  g  k  k  l  p  l  h  l  t  l  p      260
```

3/11

FIGURE 1 (CONT'D)

```
CAGGCCTTGCCTCAGTATGCTGGCTCTGGAAACCTCACCCTGGCCCTTGAAGCGAAAACA  840
----.----+----.----+----.----+----.----+----.----+----.----+
GTCCGGAACGGAGTCATACGACCGAGACCTTTGGAGTGGGACCGGGAACTTCGCTTTTGT

q  a  l  p  q  y  a  g  s  g  n  l  t  l  a  l  e  a  k  t   280
----.----+----.----+----.----+----.----+----.----+----.----+

GGAAAGTTGCATCAGGAAGTGAACCTGGTGGTGATGAGAGCCACTCAGCTCCAGAAAAAT  900
----.----+----.----+----.----+----.----+----.----+----.----+
CCTTTCAACGTAGTCCTTCACTTGGACCACCACTACTCTCGGTGAGTCGAGGTCTTTTTA

g  k  l  h  q  e  v  n  l  v  v  m  r  a  t  q  l  q  k  n   300
----.----+----.----+----.----+----.----+----.----+----.----+

TTGACCTGTGAGGTGTGGGGACCCACCTCCCCTAAGCTGATGCTGAGCTTGAAACTGGAG  960
----.----+----.----+----.----+----.----+----.----+----.----+
AACTGGACACTCCACACCCCTGGGTGGAGGGGATTCGACTACGACTCGAACTTTGACCTC

l  t  c  e  v  w  g  p  t  s  p  k  l  m  l  s  l  k  l  e   320
----.----+----.----+----.----+----.----+----.----+----.----+

AACAAGGAGGCAAAGGTCTCGAAGCGGGAGAAGGCGGTGTGGGTGCTGAACCCTGAGGCG  1020
----.----+----.----+----.----+----.----+----.----+----.----+
TTGTTCCTCCGTTTCCAGAGCTTCGCCCTCTTCCGCCACACCCACGACTTGGGACTCCGC

n  k  e  a  k  v  s  k  r  e  k  a  v  w  v  l  n  p  e  a   340
----.----+----.----+----.----+----.----+----.----+----.----+

GGGATGTGGCAGTGTCTGCTGAGTGACTCGGGACAGGTCCTGCTGGAATCCAACATCAAG  1080
----.----+----.----+----.----+----.----+----.----+----.----+
CCCTACACCGTCACAGACGACTCACTGAGCCCTGTCCAGGACGACCTTAGGTTGTAGTTC

g  m  w  q  c  l  l  s  d  s  g  q  v  l  l  e  s  n  i  k   360
----.----+----.----+----.----+----.----+----.----+----.----+

GTTCTGCCCACATGGTCCACCCCGGTTCATTAA  1113
----.----+----.----+----.----+---
CAAGACGGGTGTACCAGGTGGGGCCAAGTAATT

v  l  p  t  w  s  t  p  v  h  .   370
----.----+----.----+----.----+---
```

FIGURE 2

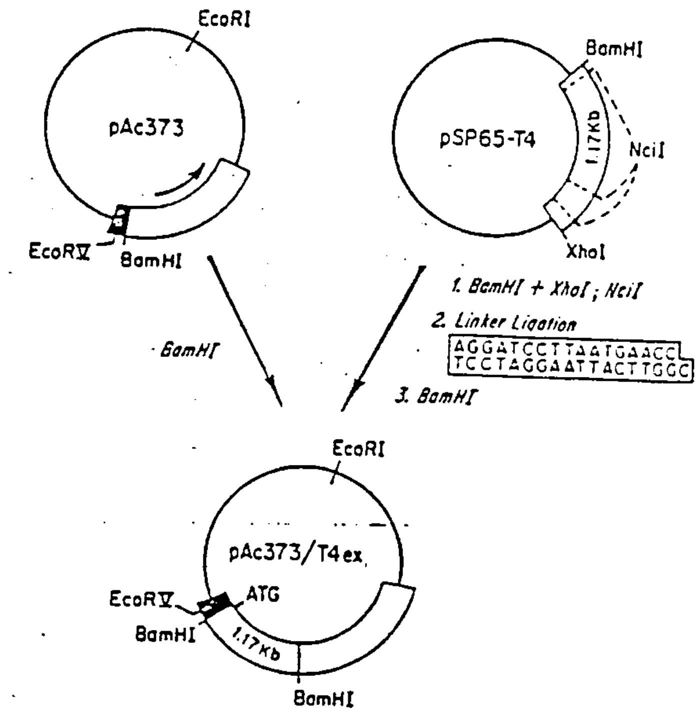
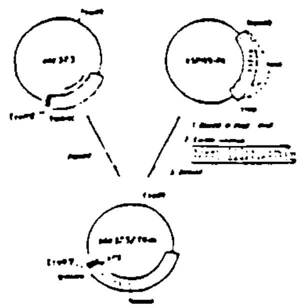

EcoRI
pAc373
EcoRV
BamHI
BamHI
pSP65-T4
1.17Kb
NciI
XhoI
1. BamHI + XhoI ; NciI
2. Linker Ligation
AGGATCCTTAATGAACC
TCCTAGGAATTACTTGGC
BamHI
3. BamHI
EcoRI
pAc373/T4ex
EcoRV
ATG
BamHI
1.17Kb
BamHI

FIGURE 3



↓

Excise T4 cDNA fragment
from pAc373/T4$_{ex}$

↓

Ligate cDNA fragment
to XbaI linkers/digest
with XbaI

↓

Ligate to Xba-cut M13

↓

Introduce into competent
TG1 host bacteria

↓

Plate/screen/select plaques
hybridizing to sense oligo-
nucleotides for use as
ss M13 templates

↓

Produce mutant oligonucleotide
(5'G-AAG-GCC-TAA-AGC-ATA-G)

↓

Kinase mutant oligonucleotide
and synthesize mutant strand
on M13 T4 template

↓

Produce ds DNA in which
both strands are mutated

↓

Excise mutated DNA from
M13 vector with Xba

↓

Ligate to Xba-cut CDM8

↓

Transfect Cos cells with
CDM8 containing mutated
T4 DNA/express CD4 protein

↓

Determine HIV-binding
ability of protein
encoded by mutated
T4 DNA

FIGURE 4

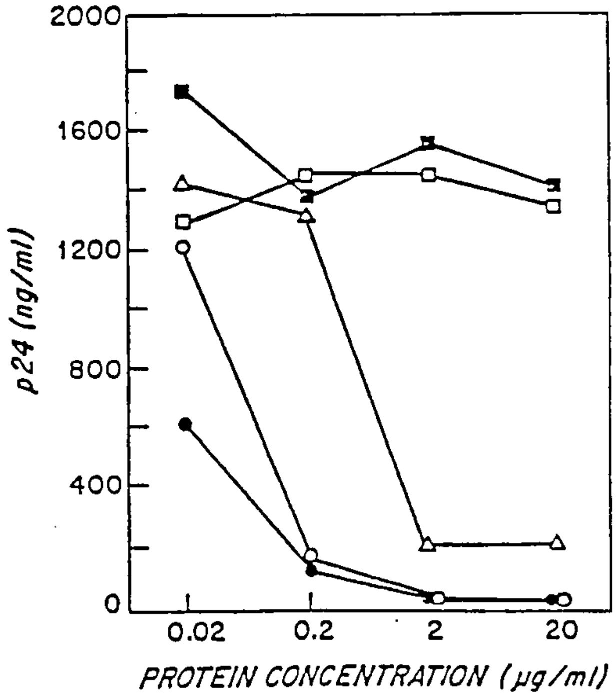

2000
1600
1200
800
400
0
p24 (ng/ml)
0.02
0.2
2
20
PROTEIN CONCENTRATION (μg/ml)

FIGURE 5

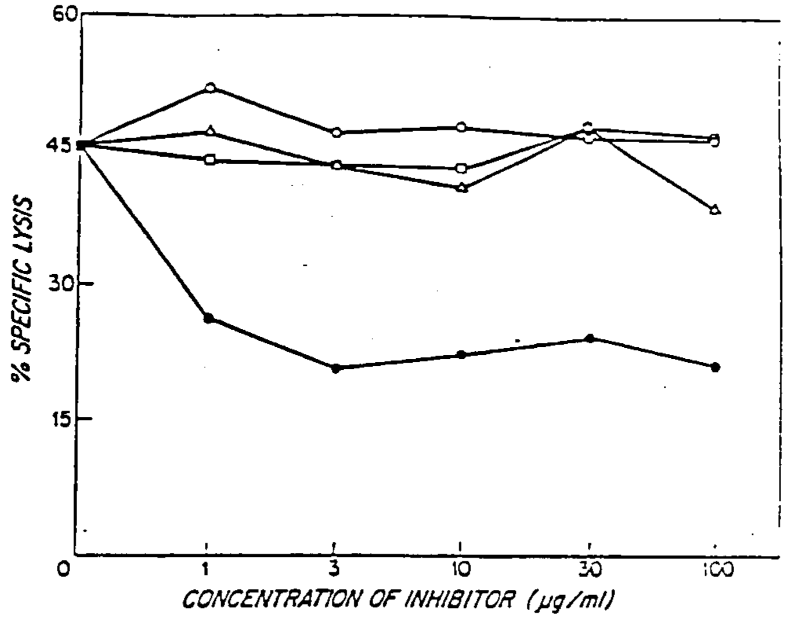


FIGURE 6

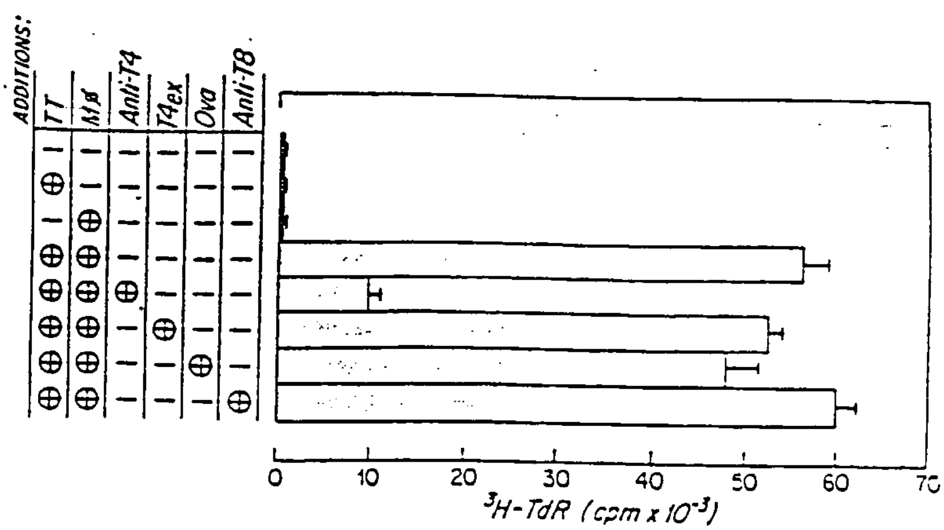


FIGURE 7A

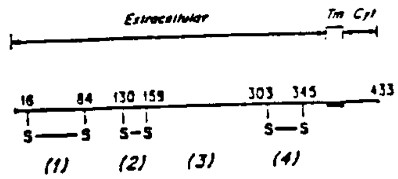


FIGURE 7B

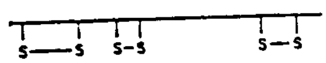


FIGURE 7C

Lys Lys Val Val Leu Gly Lys Lys Gly Asp Thr Val Glu Leu Thr 15
Cys Thr Ala Ser Gln Lys Lys Ser Ile Gln Phe His Trp Lys Asn 30
Ser Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser Phe Leu Thr 45
Lys Gly Pro Ser Lys Leu Asn Asp Arg Ala Asp Ser Arg Arg Ser 60
Leu Trp Asp Gln Gly Asn Phe Pro Leu Ile Ile Lys Asn Leu Lys 75
Ile Glu Asp Ser Asp Thr Tyr Ile Cys Glu Val Glu Asp Gln Lys 90
Glu Glu Val Gln Leu Leu Val Phe Gly Leu Thr Ala Asn Ser Asp 105
Thr His Leu Leu Gln Gly Gln Ser Leu Thr Leu Thr Leu Glu Ser 120
Pro Pro Gly Ser Ser Pro Ser Val Gln Cys Arg Ser Pro Arg Gly 135
Lys Asn Ile Gln Gly Gly Lys Thr Leu Ser Val Ser Gln Leu Glu 150
Leu Gln Asp Ser Gly Thr Trp Thr Cys Thr Val Leu Gln Asn Gln 165
Lys Lys Val Glu Phe Lys Ile Asp Ile Val Val Leu Ala Phe Gln 180
Lys Ala Ser Ser Ile Val Tyr Lys Lys Glu Gly Glu Gln Val Glu 195
Phe Ser Phe Pro Leu Ala Phe Thr Val Glu Lys Leu Thr Gly Ser 210
Gly Glu Leu Trp Trp Gln Ala Glu Arg Ala Ser Ser Ser Lys Ser 225
Trp Ile Thr Phe Asp Leu Lys Asn Lys Glu Val Ser Val Lys Arg 240
Val Thr Gln Asp Pro Lys Leu Gln Met Gly Lys Lys Leu Pro Leu 255
His Leu Thr Leu Pro Gln Ala Leu Pro Gln Tyr Ala Gly Ser Gly 270
Asn Leu Thr Leu Ala Leu Glu Ala Lys Thr Gly Lys Leu His Gln 285
Glu Val Asn Leu Val Val Met Arg Ala Thr Gln Leu Gln Lys Asn 300
Leu Thr Cys Glu Val Trp Gly Pro Thr Ser Pro Lys Leu Met Leu 315
Ser Leu Lys Leu Glu Asn Lys Glu Ala Lys Val Ser Lys Arg Glu 330
Lys Ala Val Trp Val Leu Asn Pro Glu Ala Gly Met Trp Gln Cys 345
Leu Leu Ser Asp Ser Gly Gln Val Leu Leu Glu Ser Asn Ile Lys 360
Val Leu Pro Thr Trp Ser Thr Pro Val His 370

FIGURE 8

a.

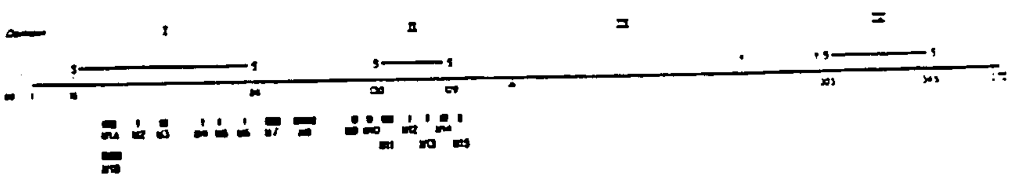


FIGURE 9

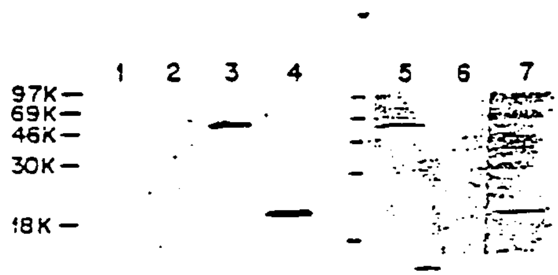
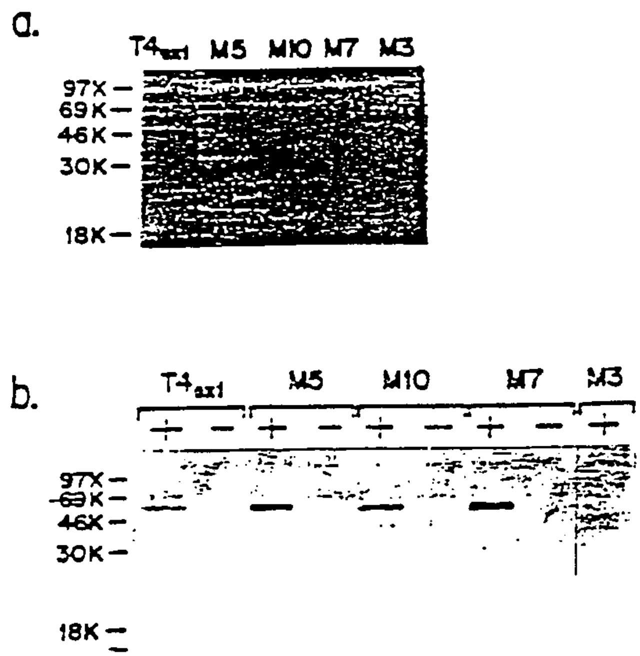


FIGURE 10


a.
T4sx1 M5 M10 M7 M3
97K
69K
46K
30K
18K
b.
T4sx1 M5 M10 M7 M3
+ − + − + − + − +
97K
69K
46K
30K
18K

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03454

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: A 61 K 37/02; C 12 N 15/00; A 61 F 5/43; A 61 B 5/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	A 61 K; C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Cell, volume 42, no. 1, August 1985, MIT, P.J. Maddon et al.: "The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene familiy", pages 93-104 see figure 3, especially fragment T4B --	1-26
X,P	WO, A, 88/01304 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 25 February 1988 see pages 69-75, examples 3-6 --	1-26
X,P	Nature, volume 331, no. 6151, 7 January 1988, (London, GB), R.E. Hussey et al.: "A soluble CD4 protein selectively inhibits HIV replication and cyncytium formation", pages 78-81 see legend to figure 1 cited in the application --	1-26

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd January 1989	1-2 FEB 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/US 88/03454

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Nature, volume 331, no. 6151, 7 January 1988, (London, GB), R.A. Fisher et al.: "HIV infection is blocked in vitro by recombinant soluble CD4", pages 76-78 see legend to figure 1	1-26

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8803454

SA 25078

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 24/01/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8801304	25-02-88	AU-A- 7879287	08-03-88
		EP-A- 0280710	07-09-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82